

RESEARCH ARTICLE

Age-related changes in intracellular cytokine expression in healthy children

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ABSTRACT. Cytokine production by human lymphocytes from healthy children (ages 0-18 years) was assessed using a flow cytometric procedure involving staining of intracellular cytokines by the paraformaldehyde-saponin procedure. To establish valid cytokine values for intracellular cytokine expression in healthy children in the different age groups, we measured 117 samples after 24 h *in vitro* stimulation with PMA, ionomycin and brefeldin followed by staining with intracellular anti-cytokine and surface antibodies. We found decreasing IL-2 expression, increasing IFN- γ and TNF- α production and stable IL-4, Ki67 and TGF β levels with advancing age. The cytokines were mainly produced by memory T-cells. Apart from age, there was a differential expression in boys and girls: boys (< 6 years) produce significantly more IL-2 ($p < 0,04$), while girls > 12 years produce more IFNg than boys of the same age ($p < 0,05$). This systematic analysis of cytokine profiles during childhood allows a better understanding of immune maturation and will contribute significantly to the interpretation of cytokine data from children with pathological conditions.

Keywords: cytokine expression, children, age changes, intracellular

T cells are important regulatory cells of the immune system. Many of their functions are mediated by the expression and secretion of cytokines [1]. An imbalance in cytokine production profiles has been demonstrated in several clinical conditions and in pathophysiological mechanism in certain immunological diseases, for example acute GVHD, HIV and several autoimmune diseases [2-4]. Furthermore, there are several clinical studies which have demonstrated that cord blood stem cell transplantation is associated with a lower incidence or decreased severity of GVHD [1, 4, 5]. It has been hypothesized that one central reason for this observation may be decreased cytokine expression in cord blood T cells [6, 7].

However, data involving normal cytokine production profiles for the neonatal period onwards in healthy infants, and for children and adolescents are lacking. This information however, is important not only for the further elucidation of normal, immunological variations among the different age groups, but also for studies on cytokine networks in pathological conditions in children with a variety of autoimmune, infectious or transplant-associated diseases.

Intracellular staining of cytokines using flow cytometry is a method that allows the simultaneous staining of cyto-

kines and surface markers. It thus permits identification of cytokine production in subpopulations without prior cell sorting and without the use of clones at a single cell level [8-10]. Lymphocytes can be subdivided into naïve and memory cells, the latter having been primed by an antigenic stimulus [11]. These subpopulations can be identified by their expression of variant CD45 isoforms. Naïve cells carry the CD45RA isoform while memory cells express the CD45RO epitope [12]. Memory cells are expected to mount a secondary immune response to a previously encountered antigen faster and more effectively than non-primed lymphocytes. Our systematic analysis, from birth until adolescence, is the first to include more than 100 healthy children, and provides an insight into the development of cytokine expression in lymphocyte subpopulations during childhood.

DONORS AND METHODS

Materials

Antibodies to the surface epitopes CD3 (clone UCHT1), CD4 (RPA-T4), CD8 (RPA-T8), CD16 (3G8)/56 (B159),

CD19 (HIB19), CD45RA (HI100), CD45RO (UCHL1), Ki67 (B56), TGF β (TB21), TNF α (Mab11), IFN γ (B27), IL2 (MQ1-17H12) and IL4 (MP4-25D2) were all purchased from BD (Heidelberg, Germany). Paraformaldehyde, PBS, saponin, PMA, ionomycin and brefeldin, as well as HEPES buffer, were obtained from Sigma (Taufkirchen, Germany). RPMI medium was a product of Seromed biochrome (Berlin, Germany).

Methods

Heparinized blood was obtained from healthy children; 1×10^6 cells/ μ L were suspended in RPMI 1640 (with 2.0 g/L NaHCO₃) containing 10% fetal calf serum, 1% glutamine and 1% penicillin/streptomycin. Cells either were left unstimulated or were stimulated with PMA (10 ng/mL) and ionomycin (1 μ M) for 24 h at 37°C and 5% CO₂. In order to promote the accumulation of *de novo* synthesised cytokines in the Golgi apparatus of the synthesising cells, brefeldin was added to the cells at a concentration of 2.5 μ g/mL. After stimulation, cells were processed as described by Jung *et al.* [8]. Briefly, cells were harvested, washed once in HBSS, stained with surface markers for 10 min in a dark room at room temperature and then fixed for 10 min with 4% paraformaldehyde. Cells were washed twice, resuspended in saponin-buffer (HBSS containing 0.1% saponin and 0.001 M HEPES buffer). Cells were stained with directly conjugated, anti-cytokine antibodies. Cells were incubated for 20 min at 4°C in the dark, washed once in saponin buffer and finally resuspended in HBSS. Cytokine production was determined in CD3 $^+$, CD8 $^+$, CD4 $^+$, CD45RA $^+$ and CD45RO $^+$ cells using a BD four colour Calibur flow cytometer. Several controls were performed to prove the specificity of intracellular cytokine staining. Thresholds for cytokine positivity were set using unstained cells as a control for non-specific bonds. Results for cytokine positive cells (mean \pm SD) are expressed as a percentage of the respective subpopulation. A minimum of 10 000 lymphocyte-gated events was acquired in list mode and analysed with cell quest software (Becton Dickinson). Events were gated on lymphocytes via forward and side scatter and for CD3 $^+$, CD3 $^+$ CD4 $^+$ and CD3 $^+$ CD8 $^+$ cells or CD3 $^+$ /CD45RA $^+$ /CD45RO $^-$ and CD3 $^+$ /CD45RA $^-$ /CD45RO $^+$.

Statistical analysis

Students' paired t test for mean differences was used to analyze data for levels of statistical significance among the three age groups. Correlation between surface isotope expression and cytokine production was assessed using the Pearson correlation coefficient. In all statistical applications $p < 0.05$ was considered significant.

Patients

Inclusion criteria for this study were healthy children of Caucasian origin who had no history of chronic disease, no family history of immune-mediated disease, no sign of acute or chronic infection and who had not received any medication within the preceding week that could have influenced immune function. The children were immu-

nized according to the recommended immunisation schedule for children of the German Standing Committee on Vaccination (STIKO).

In total, 117 samples were obtained. Age groups were constructed to represent age-related changes in cytokine expression. The distribution in the groups was as follow: group 1 (cord blood): 25 samples (14 boys, 11 girls); group 2 (< 2 y): 21 samples (13 boys, 8 girls, median age 212 days [0.6 years]); group 3 (2-6 y): 25 samples (14 boys, 11 girls, median age: 1420 days [3.9 years]); group 4 (6-12 y): 23 samples (15 boys, 8 girls, median age: 2 777 days [7.6 years]); group 5 (12-18 y): 23 samples (12 boys, 11 girls, median age: 5076 days [13.9 years]).

The study was approved by the Human Subjects Committee of our institution (Study # 133/04, 9/28/07, 11/25/05, 12/13/04). Patients and guardians participating in this study gave informed consent according to institutional guidelines following the Declaration of Helsinki.

RESULTS

Cytokine-producing cells in CD4 $^+$ and CD8 $^+$ T cell subsets

To define normal ranges for intracellular cytokine expression in T-cells in childhood, a total of 117 subjects were studied. The median results are presented in *table 1* for each age group. In cord blood, a large number of CD3 $^+$, CD4 $^+$ and CD8 $^+$ cells produce IL-2, whereas IFN- γ production was low in all cord blood T cells. Except for absolute IL-2-production, there was an obvious general trend towards increasing cytokine expression with increasing age. We found a significant positive correlation index between age and cytokine production for IFN- γ , TNF- α and IL-4 in CD3 $^+$ cells (*figure 1*), for TNF- α and IFN- γ in CD8 $^+$ cells (*figure 2*) and for IFN γ in CD4 $^+$ cells. It is also worth noting that we found an increasing population producing both IL-2 and IFN- γ at the single cell level. The expression of TH2-cytokines such as IL-4 was comparatively low and did not reach significance. Double staining cytokine expression of IL-4 and TH1 cytokines remained stable and low in the different age groups.

In summary, we found decreasing IL-2 expression in T cells (CD4 and CD8 subset), increasing IFN- γ and TNF- α production (*figure 1,2*) and stable IL-4, Ki67 and TGF- β levels [data not shown], with increasing age.

Cytokine production profiles in CD45RO and CD45RA T cells

Aware of the fact that CD45RA and CD45RO cells might produce different cytokines, we directly analyzed the expression of CD45 isotypes. In all age groups, IL-2 was mainly produced by CD4 cells, the relative contribution of naïve and memory cell population being approximately equal. IFN- γ was mainly expressed by CD8CD45RO cells, whereas TNF- α was mainly expressed in CD4CD45RO cells, and IL4 expression was similar in memory CD4 and CD8 cells. At birth,

Table 1

Normal range of intracellular cytokine production by T lymphocytes in different age groups. Results are shown as mean of percentage cytokine-positive cells of the respective subpopulation \pm standard deviation. Absolute count shown in [...]

	Cord blood (group 1; n = 25)	0-2 years (group 2; n = 21)	2-6 years (group 3; n = 25)	6-12 years (group 4; n = 23)	12-18 years (group 5; n = 23)
CD3 (% of total lymphocyte count)	55 \pm 9	75 \pm 8	68 \pm 8	71 \pm 8	71 \pm 6
Lymphocyte (% and [μ L] of total leukocyte count)	41 \pm 5 [5 400]	47 \pm 8 [4 100]	46 \pm 6 [3 600]	40 \pm 4 [2 400]	32 \pm 5 [2 100]
CD3 IL-2 [μ L]	28 \pm 5 [1 520]	27 \pm 8 [1 107]	33 \pm 7 [1 180]	40 \pm 7,5 [1 056]	44 \pm 7,5 [924]
CD3 IFN- γ [μ L]	4 \pm 1 [124]	15 \pm 9,5 [615]	15 \pm 5,5 [540]	22 \pm 7,5 [528]	24 \pm 6 [504]
CD3 TNF- α [μ L]	7 \pm 3,5 [217]	15 \pm 6 [615]	21 \pm 4,5 [756]	22 \pm 8,5 [528]	32 \pm 6,5 [672]
CD3 IL-4 [μ L]	0,2 \pm 0,5 [6]	0,7 \pm 1 [28]	1 \pm 1 [30]	1 \pm 0,5 [24]	1 \pm 1 [21]
CD4 IL-2	71 \pm 12	14 \pm 8,5	29 \pm 9	26 \pm 7	37 \pm 10
CD4 IFN- γ	4 \pm 3,5	8 \pm 3	11 \pm 6,5	13 \pm 6	17 \pm 7,5
CD4 TNF- α	4 \pm 2	17 \pm 3	22 \pm 9	24 \pm 6	25 \pm 5
CD4 IL-4	0,6 \pm 0,5	1 \pm 1	1 \pm 1	1 \pm 2	1 \pm 1
CD8 IL-2	50 \pm 9	18 \pm 12	18 \pm 9	25 \pm 8	27 \pm 7,5
CD8 IFN- γ	9 \pm 3	21 \pm 10,5	13 \pm 9	15 \pm 7	22 \pm 6,5
CD8 TNF- α	7 \pm 4	21 \pm 3,5	20 \pm 7	26 \pm 7,5	23 \pm 6,5
CD8 IL-4	1 \pm 1	1 \pm 2,5	2 \pm 1	1 \pm 0,5	1 \pm 1

the memory T cell subset is very rare, but with increasing age the expression by CD45RO progressively and significantly increases so that in adults the majority of T cells display a memory phenotype. A significant positive correlation between CD45RO cells and IFN- γ production was found in both CD4 and CD8 positive cells (not shown).

Different cytokine expression with respect to gender

In addition to age-related changes in cytokine expression during childhood, we compared cytokine production in male and female children. Careful attention was paid to the balance of females and males in each group. Interestingly, we found higher IL-2 expression ($p < 0.04$) in males in age groups 1, 2 and 3 (age < 6 y). With increasing age, we detected a reverse ratio in females, with increased values of IL-2 in group 4 ($p < 0.01$), and of IFN- γ in group 5 ($p < 0.04$), compared to males.

DISCUSSION

Intracellular cytokine detection by FACS analyses is increasingly used as a human immune status indicator. There are different studies that have looked at cytokines in healthy, elderly people [13], in neonates [6, 14] as well as in patients with underlying diseases, for example GVHD, autoimmune or atopic conditions [1, 4, 5]. However, there are only a few studies, with relatively small cohorts ($n < 50$), that took a closer look at changes in cytokine expression during childhood [7, 15]. These investigators found a trend to lower cytokine production in very young children [6, 7, 15].

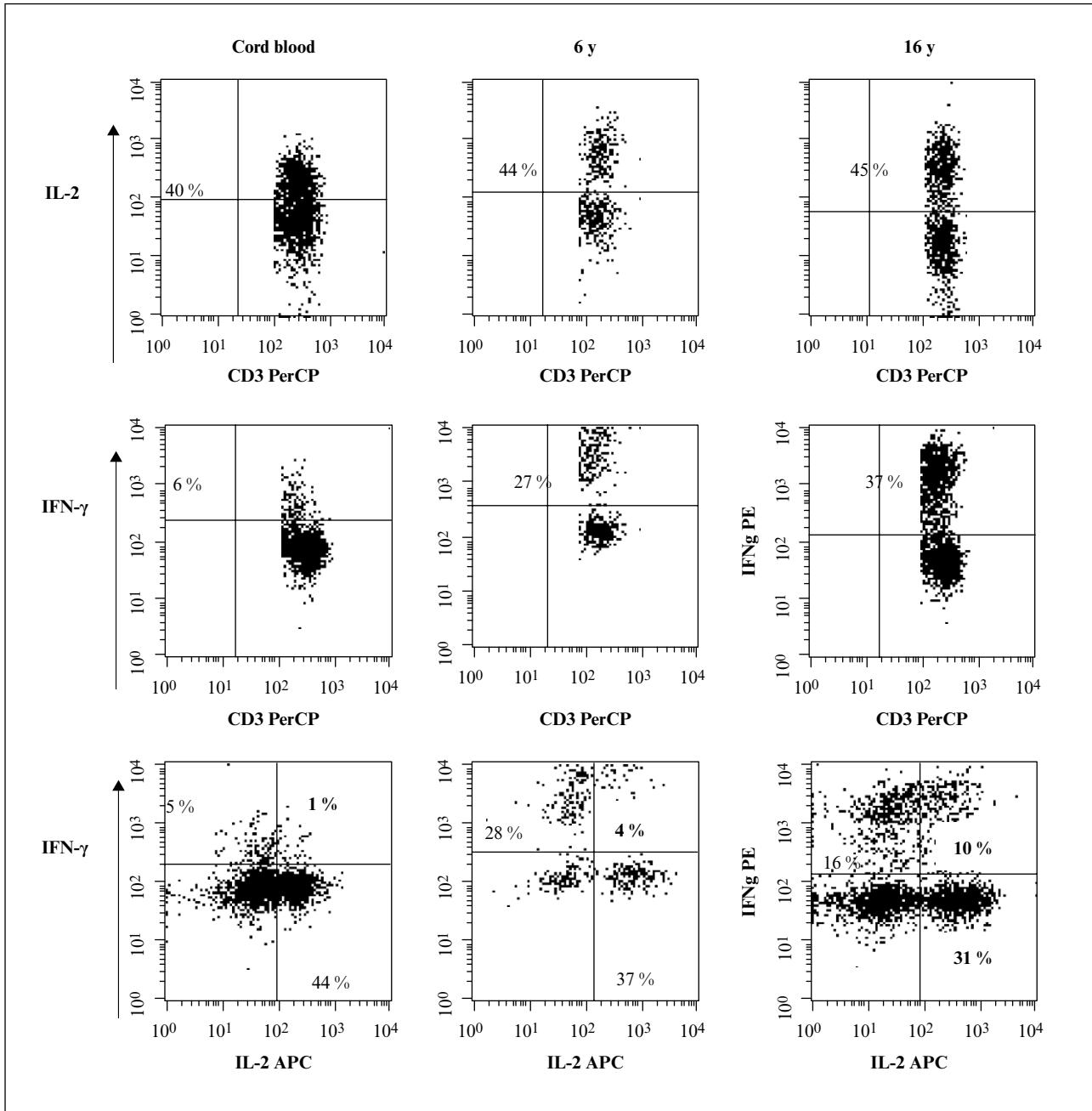
The method for induction of intracellular cytokine expression is well known [8, 9]. Mascher [16] performed kinetic and distribution studies in adults, which con-

firmed that stimulation between 20 and 24 h seemed to be a good time frame for obtaining a stable level of Th1 and Th2 cytokines; furthermore they showed that cytokines are mainly expressed by memory T cells.

As expected, we were able to demonstrate that neonates show, almost exclusively, T cells with a naïve phenotype (data not shown [17]), the expression of the memory phenotype (expression of CD45R0) enhancing with increasing age. As most cytokines are predominantly expressed by memory cells, we found higher cytokine levels in older children [6, 18]. In neonates, the naïve T cell predominantly produces IL-2, which is in accordance with the existence of the naïve, Th0-Type T cell. This cell produces IL-2, and, via an auto-stimulatory route, participates in the maturation, differentiation and production of T cells.

With the exposure to antigens in the environment in early infancy and childhood, as demonstrated by the typical viral infections during this phase, or the production of antibodies against antigens encountered through vaccination, the immune system starts to build memory T and B cells. In infancy, while B cells start producing antigen-specific antibodies and immunoglobulin titers rise, the development of memory T cells is demonstrated by an increasing expression of effector-T cell cytokines such as IFN- γ and TNF- α . Also, during the production of T cells, the cells develop either a Th1 phenotype (production of IFN- γ) or a Th2 phenotype (IL-4). As production of Th2-Type cytokines such as IL-4, IL-5 or IL-10 is generally very low, we did not see any trend with advancing age.

We cannot exclude an influence of breast feeding versus formula diet in respect to cytokine expression, especially in the very young children. However, we did not look at this and there are only very few data regarding this topic [19, 20]. However, this might be interesting for future investigations.

**Figure 1**

Changes in Th1 cytokine production by T cells in different age groups (%).

In this figure we show changes in the Th1 cytokine expression at different ages. The first row shows IL-2 expression in CD3 positive T cells, the second row shows IFN- γ expression in CD3 positive T cells; the third row shows the double staining of Th1 cytokines for IL-2 and IFN- γ in CD3 positive T cells.

When analyzing intracellular cytokine staining after stimulation with PMA, ionomycin and brefeldin A in pediatric patients, we found a positive, significant correlation with age and increasing IFN- γ values in both helper and cytotoxic T cells. This corresponds with findings of increased Th1 levels in older children described by Chipeta, and Hoffman [7, 15]. Even though Berdat [21] could not confirm a significant increase in TNF- α serum levels, we were able to demonstrate a significantly higher expression in CD3 $^+$ and CD8 $^+$ cells in older children by intracellular cytokine staining. However, we could not find the correlation in CD4 $^+$ cells described by Hoffmann [15].

The significance of the changes in the Th1-Th2 balance with age remains unclear. A shift from a predominant Th1 level in young individuals towards a Th2 cytokines in the elderly has been recently described [18]. We found higher, absolute cytokine expression in Th1 and Th2 cells generally in older children, with a Th1 predominance. Interestingly, girls older than 12 years showed a significantly higher IFN- γ production than boys of the same age. In the same age group, the higher prevalence of autoimmune disease in females is well recognised. Since a predominance of Th1-cytokines has been demonstrated for many autoimmune diseases [2, 3], it may be

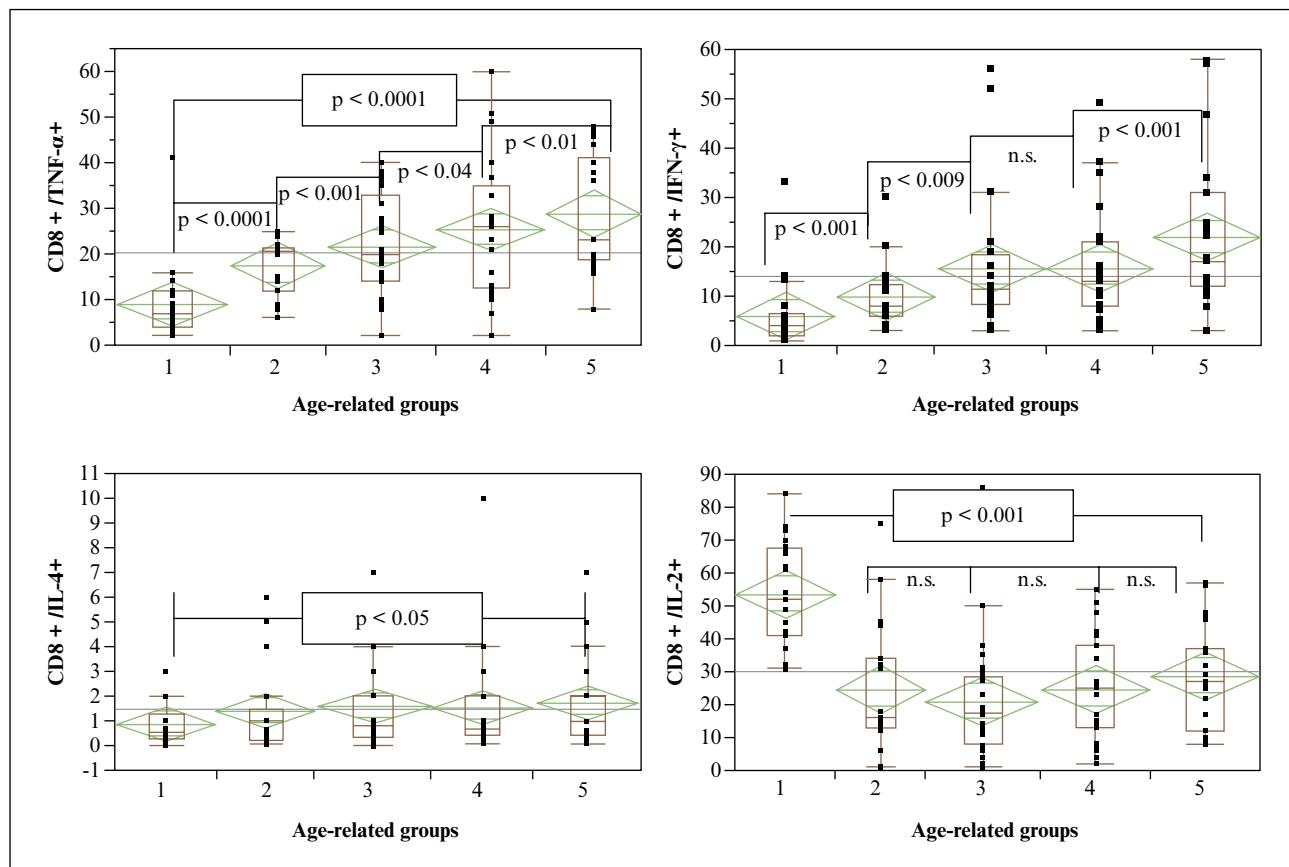


Figure 2

Intracellular cytokine production by T Lymphocytes from healthy children. Box plots represent cytokine expression in $CD3^+CD8^+$ cells (quantiles: 10%, 25%, median, 75%, 90%). Cytokine expression in $CD3^+$ and $CD3^+CD4^+$ T cells is also shown. The red square shows the 25% quantile, the median in the middle and the upper limit indicates the 75% quantile. The mid-line shows the mean.

hypothesized that the higher production of IFN- γ in females might be a contributing, genetic factor.

In summary, the aim of this study was to establish valid cytokine values for intracellular cytokine expression in healthy children using a common, easily reproducible method. We were able to demonstrate an association between age and cytokine expression, and have established age-specific tables of normal cytokine expression values.

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