

In vitro cytokine production and phenotype expression by blood mononuclear cells from umbilical cords, children and adults

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Age related differences in immunological reactions include variations in the *in vitro* functions of blood mononuclear cells (MNC). In an attempt to understand the mechanism behind these differences we examined age related differences in the phenotype profiles of MNC in parallel with the *in vitro* production of interleukin IL-6, tumour necrosis factor alpha (TNF α) and interferon gamma (IFN γ) in neonates, children and adults. In cultures without added polyclonal activators IL-6 and TNF α levels in children were 3–6 times higher than those of umbilical cords and adults. However, using optimal *in vitro* stimulation (*E. coli* lipopolysaccharide (LPS), phytohaemmagglutinin or pokeweed mitogen (PWM)) no significant differences in the levels of these cytokines were observed. The levels of IFN γ in PWM driven cultures followed a different pattern with comparable levels in children and adults, and unmeasurable levels in cord blood MNC. Flow cytometry analysis of the phenotypic distribution of MNC revealed age related differences in the expression of CD3, CD4, CD8, CD14, CD19, CD45RA, CD45R0, CD2, LFA-1, ICAM-1 and LFA-3. Correlation studies did not indicate that the observed differences in cytokine production could be ascribed to differences in the frequency of monocytes, T cells or B cells. The TNF α levels in suboptimally stimulated cultures correlated negatively with the expression of LFA-3 and positively with CD45RA, while IFN γ correlated positively with CD2, LFA-1, CD45R0 and CD8.

In conclusion, the study provides evidence of age related differences in the production of TNF α , IL-6 and IFN γ among neonates, children and adults. These differences may to some extent be caused by differences in the expression of cell surface molecules involved in cellular interactions and signalling.

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Immunological and inflammatory reactions are known to vary with age. Human newborns have increased susceptibility to disseminated viral and bacterial infections compared with children. Although the mechanisms behind these differences are not fully understood, impairment of lymphocyte functions may play a major role. Thus, stimulation of mononuclear cells (MNC) from neonates induces low-level antibody responses which are restricted to the IgM isotype (1–3).

Activation of lymphocytes and B cell maturation is dependent upon the interaction between cell surface receptor molecules as well as the production of soluble factors, cytokines, by the mononuclear cells.

Interleukin (IL) -1, IL-6 and tumour necrosis factor alpha (TNF α) are products of activated monocytes/macrophages, which are critically involved in lymphocyte activation as well as local and systemic inflammatory reactions. Further activation and maturation of lymphocytes is regulated through the production of a secondary wave of cytokines, including IL-2 and interferon gamma (IFN γ) released by T cells (4). Although cord blood IL-2 production is normal (5, 6) the production of interferon gamma (IFN γ) has been found to be markedly reduced at the protein level (5, 7) and at the level of mRNA (8, 9).

Our knowledge on age dependent variations in cytokine production by monocytes is scant. Studies

comparing IL-1, TNF α and IL-6 production in neonates and adults have led to contrasting results (10–13), while the production of these cytokines in children has been less extensively investigated (14).

Interactions between various lymphocyte subpopulations and monocytes are mediated by the antigen receptor-CD3 complex as well as accessory surface molecules such as CD4 and CD8. Furthermore, adhesion receptors, including LFA-1, LFA-2 (CD2), LFA-3 and ICAM-1 are thought to contribute to the immune response by mediating adhesion as well as signalling (15).

The purpose of the present study was to compare the *in vitro* production of IL-6, TNF α and IFN γ as well as the phenotype profiles of mononuclear cells in children with those of neonates and adults and to look for correlations between the cytokine profiles and the flow cytometric distribution of the cells.

Material and methods

Blood sources

Newborn MNC were separated from heparinized cord blood, collected from nine umbilical cords. Heparinized, venous blood was obtained from 45 healthy children (24 males, 21 females) with a median (range, quartiles) age of 9.0 years (0.6–16.5, 6.1–12.0) and from 20 healthy adults (eight males, 12 females) aged 36.5 years (26.5–54.4, 29.1–47.5). Individuals with recent infections (within 3 weeks) and individuals undergoing medical treatment were excluded from the study.

Mononuclear cells

Mononuclear cells (MNC) were obtained by gradient centrifugation of heparinized peripheral blood on Ficoll-Hypaque (LymphoprepR; Nyegaard, Oslo, Norway). The cells were washed and resuspended in RPMI 1640 (Gibco, Paisley, Scotland), supplemented with a pool of 10% heat inactivated human serum pool, L-glutamine (0.8 mM), penicillin and streptomycin. All reagents, including RPMI and the serum pool were tested by a chromogenic limulus amoebocyte lysate assay (OCL-1000; Whittaker Bioproducts Inc., Walkerville, MD, USA), and found to contain less than 50 pg/ml *E. coli* lipopolysaccharide equivalent.

Cytokine production

For the generation of cytokine rich supernatants 10^6 MNC/ml were cultured at 37°C in polypropylene plastic tubes (Nunc, Roskilde, Denmark) in a humidified atmosphere containing 5% CO $_2$. The cultures were stimulated with *E. coli* lipopolysaccharide

(LPS), 1 μ g/ml (Difco, Detroit, MI, USA), phytohemagglutinin-P (PHA) (Difco) (20 μ g/ml) or pokeweed mitogen (PWM) (0.8 μ g/ml) (Gibco), and the supernatants were harvested after 24 h (LPS, PHA) or 48 h (PWM). These stimulation protocols were designed according to initial experiments in which the kinetics and stimulatory requirements for IL-6, TNF α and IFN γ were studied. Although significant production of IFN γ was observed in most 24h PHA cultures, IFN γ production was optimal in 48h PWM cultures. TNF α production was optimal in 24h LPS cultures, followed by PHA 24h - and PWM 48 h cultures. The opposite pattern was found for IL-6. In order to be able to compare IL-6 and TNF α , which are closely related cytokines, these were measured in all three sets of cultures, while IFN γ was examined only in PWM cultures at 48 h. Cell viability examined by trypan blue exclusion at 48 h exceeded 80%.

Flow cytometry

The following FITC or PE-conjugated monoclonal antibodies were employed: Dako, Glostrup, Denmark: CD2 (FITC), T3 (PE) (CD3) T4 (PE) (CD4), T8 (FITC) (CD8), CD19 (PE), CD25 (PE), UCHL-1 (PE), (CD45R0), LFA-1 (FITC), ICAM-1 (PE). Becton Dickinson (Mountain View), CA, USA: HLADR (FITC), Leu-18 (FITC) (CD45RA), Leu-M3 (FITC) (CD14). The negative controls were isotype matching mouse IgG antibodies (FITC or PE) (Becton Dickinson). LFA-3 was measured using unconjugated mouse LFA-3 antibody (Immunotech, Marseille, France) in combination with F(ab) $_2$ (FITC) (Dako). The cells were washed, adjusted to 10^7 /ml in phosphate-buffered saline (PBS) with 5% heat inactivated fetal calf serum, and incubated with the antibodies for 30 min at 4°C. After 3 washes, the fluorescent staining was analyzed using a FACStar flow cytometer (Becton Dickinson). The forward and side scatters were used to gate out dead cells.

ELISA for human cytokines

IL-6, TNF α and IFN γ were measured by double sandwich ELISAs using monospecific, polyclonal rabbit antibodies to purified recombinant cytokines (16). Immuno-Maxisorp plates (Nunc, Roskilde, Denmark) were coated with protein-A affinity-purified IgG. Non-attached sites were blocked with 5% human serum albumin in PBS. Biotinylated rabbit antibodies were used as detecting antibodies along with streptavidin-peroxidase (Kirkegaard and Perry La., Gaithersburg, MD, USA). Development was carried out with 1,2-phenylenediamine dihydrochloride and measured at 492 nm. The intra- and interassay coefficients of

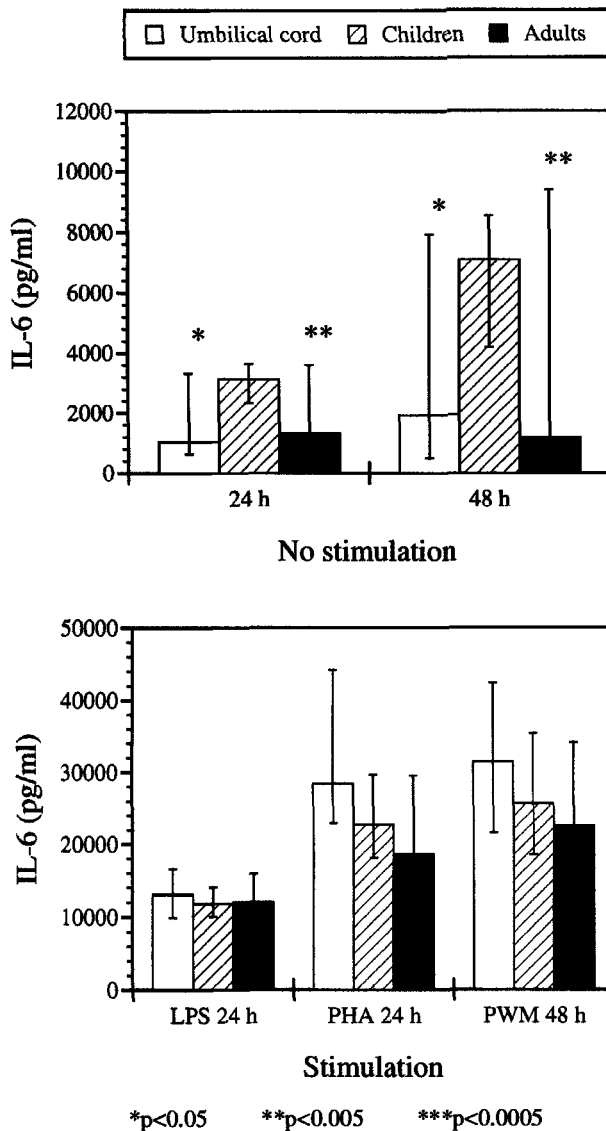


Fig 1. Supernatant levels of IL-6 at 24 h and 48 h in cultures of mononuclear cells from umbilical cords (n=9), children (n=44) and adults (n=20). Upper panel: no stimulation. Lower panel: stimulation with *E. coli* lipopolysaccharide (LPS), phytohaemagglutinin (PHA) or pokeweed mitogen (PWM). Results are medians and quartiles. Blank box: cord blood; hatched box: children; filled box: adults. * $p<0.05$, ** $p<0.005$.

variation were less than 15%. The assays were calibrated with international standards of the respective cytokines (National Institute for Biological Standards and Controls, Potters Bar, Hertfordshire, UK). The standards are supplied as stated amounts and assigned unitage. The sensitivity of these ELISAs were 8 pg/ml (TNF α), 30 pg/ml (IL-6) and 0.3 U/ml (IFN γ).

Statistics

The Mann-Whitney test was used for the comparison of cytokine data and flow cytometric data

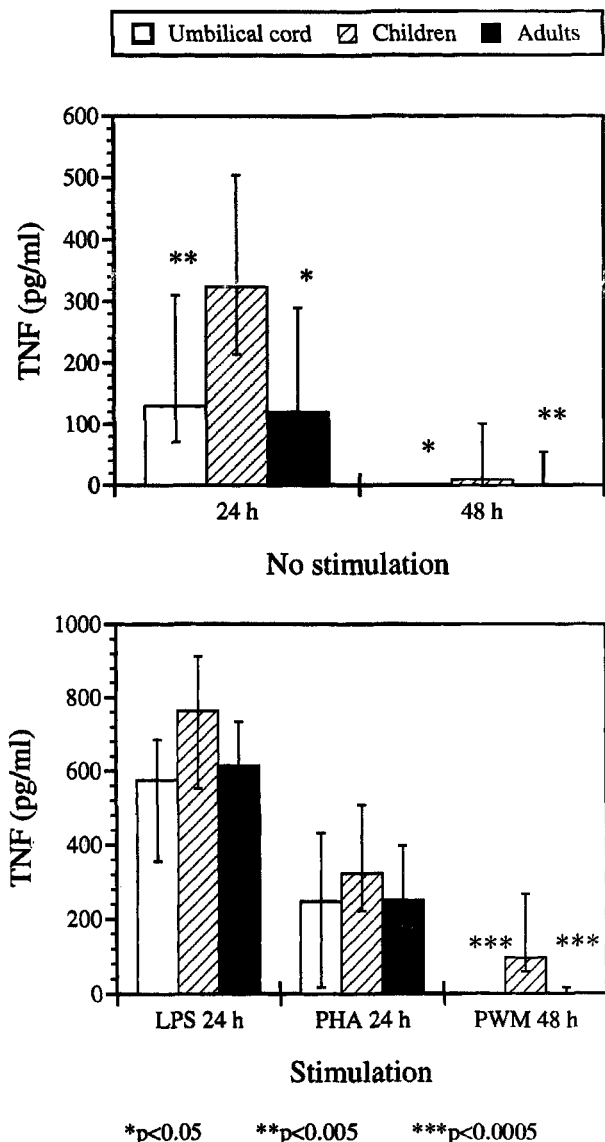


Fig 2. Supernatant levels of TNF α at 24-h and 48-h in cultures of mononuclear cells from umbilical cords (n=9), children (n=44) and adults (n=20). Upper panel: no stimulation. Lower panel: stimulation with *E. coli* lipopolysaccharide (LPS) phytohaemagglutinin (PHA) or pokeweed mitogen (PWM). Results are medians and quartiles. Blank box: cord blood; hatched box: children; filled box: adults. * $p<0.05$, ** $p<0.005$, *** $p<0.0005$.

of different age groups. The Spearman test was used for correlation analysis.

The level of significance was chosen at 5%. The study was approved by the regional ethics committee.

Results

Cytokine levels

The supernatant levels of IL-6 and TNF α were measured in 24- and 48-h cultures. In unstimulated cultures there was a moderate but significant IL-6

Table 1. Surface antigen expression by mononuclear cells in cord blood, children and adults. Results are given as percent pos. cells (medians and quartiles).

	Cord blood (n=9)	Children (n=43)	Adults (n=20)
CD3	44 (37–51) ^{f,h}	69 (64–73) ^f	64 (33–77) ^f
CD4	38 (33–44) ^{f,g}	44 (40–51) ^f	46 (39–51) ^f
CD8	15 (13–18) ^{f,h}	26 (22–29) ^f	25 (19–29)
CD14	11 (6–15) ^f	7 (5–9) ^f	7 (5–12)
CD19	9 (6–10)	11 (8–15) ^j	7 (6–12) ^j
CD45RA	65 (56–74) ^f	79 (73–81) ^{k,l}	62 (55–68) ^j
CD45RO	16 (11–19) ^{f,h}	27 (23–34) ^{k,l}	37 (25–45) ^{j,i}
HLA-DR	8 (5–12)	10 (8–13)	9 (8–16)
CD25	4 (3–4)	3 (3–5)	3 (1–4)
LFA-1	76 (70–82) ^{j,i}	94 (91–97) ^j	95 (89–96) ⁱ
ICAM-1	5 (3–9) ^f	5 (3–8)	2 (1–5) ^f
LFA-3	39 (32–56)	35 (29–48) ^f	49 (40–56) ^f
CD2	47 (38–59) ^{k,i}	76 (70–78) ^f	71 (66–78) ⁱ

Significant differences : cord blood versus children: a: $p<0.05$, b: $p<0.005$, c: $p<0.001$, d: $p<0.0005$, e: $p<0.0001$, f: $p<0.00001$.

Cord blood versus adults : g: $p<0.05$, h: $p<0.005$, i: $p<0.0005$.

Children versus adults : j: $p<0.05$, k: $p<0.005$, l: $p<0.00001$.

production, particularly in children, where the median levels were 3–6 times higher than those of neonates and adults (Fig. 1). The TNF α levels followed the same pattern (Fig. 2).

In cultures stimulated with LPS, PHA or PWM the highest TNF α levels were again found in children (Fig. 2). The differences between the three groups did only reach significance in the case of PWM stimulation. The IL-6 levels in the stimulated cultures reached comparable concentrations in the three age groups (Fig. 1).

The levels of IFN γ in PWM stimulated cultures from neonates were below the detection limit, but reached comparable levels in children and adults (Fig. 3). The levels of IFN γ in unstimulated cultures were generally close to the detection limit (Fig. 3).

The IFN γ levels in PWM stimulated cultures correlated with TNF α levels in PWM cultures ($r=0.453$, $p<0.0001$) as well as TNF α levels in 24- and 48-h unstimulated cultures ($r=0.328$, $p<0.05$ and $r=0.250$, $p<0.05$, respectively). IFN γ levels also correlated with IL-6 levels in 24- and 48-h unstimulated cultures ($r=0.245$, $p<0.05$ and $r=0.232$, $p<0.05$, respectively).

No significant differences in the levels of IL-6, TNF α and IFN γ were found between children younger than 10 years and children older than 10 years (not shown).

Phenotypic distribution of MNC

We looked for differences in the distribution of MNC subsets, including CD3, CD4, CD8, CD14, and CD19 (reacting with antigens expressed by a majority of T cells, helper T cells, cytotoxic T cells, monocytes, and B-cells, respectively) (Table 1). The

frequencies of both CD3, CD4 and CD8 positive cells were reduced in neonates compared with children and adults, whereas the percentage of CD14 positive cells was increased in neonates compared with children. The median frequency of CD19 positive cells appeared to be highest in children, although the difference towards neonates was insignificant.

The expression of two variants of the leucocyte common antigen was examined. CD45RA, which is preferentially expressed by "naive T cells", correlated negatively with age ($r=-0.370$, $p<0.002$), while the reciprocal subset, CD45RO, expressed by "memory T cells", correlated positively with age ($r=0.539$, $p<0.00001$). The CD25 (p55 IL-2 receptors) and HLA-DR were expressed to a similar degree in the three groups.

The expression of the examined cellular adhesion molecules, LFA-1, ICAM-1, LFA-3 and CD2, appeared to vary in relation to age (Table 1). Thus, the frequencies of LFA-1 and CD2 were lower in neonates than in children and adults, while ICAM-1 was higher in neonates than in adults. LFA-1, CD2 and LFA-3 correlated with CD45RO ($r=0.529$, $p<0.00001$; $r=0.499$, $p<0.00005$; and $r=0.399$, $p<0.001$, respectively).

Correlations between phenotypes and cytokine production: The levels of TNF α and IL-6 in 48 h unstimulated cultures correlated weakly with the expression of LFA-3 ($r=-0.283$, $p<0.02$ and $r=-0.241$, $p<0.05$, respectively). In addition TNF α correlated with the expression of CD45RA ($r=0.360$, $p<0.002$ (24 h unstimulated), $r=0.230$, $p<0.05$ (48 h unstimulated) and $r=0.409$, $p<0.0005$ (PWM cultures)) (Fig. 4). IL-6 and TNF α did not correlate with the expression of CD14.

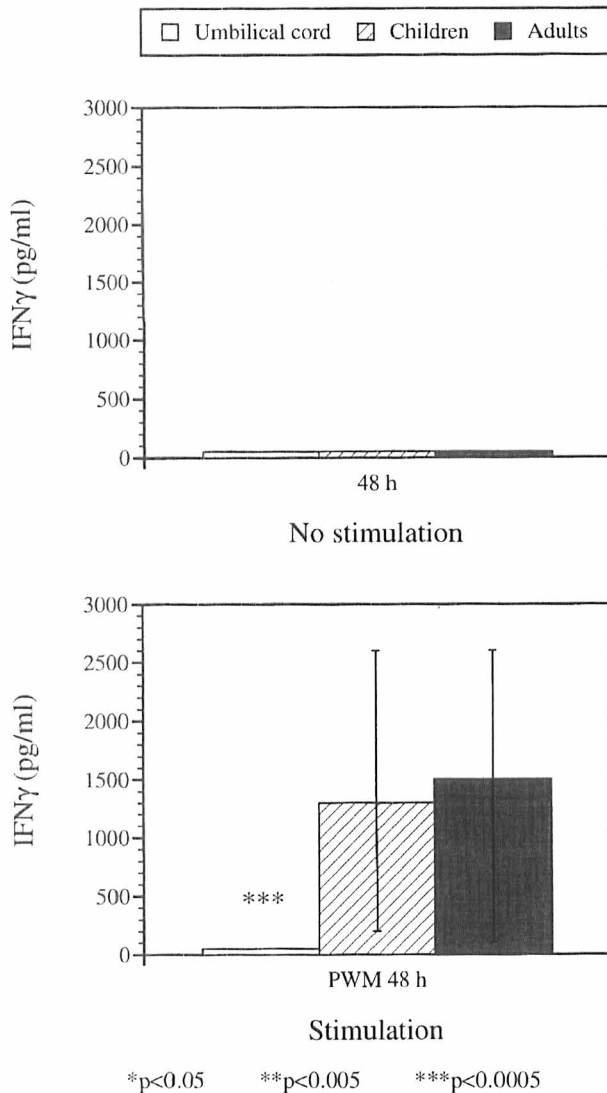


Fig. 3. Supernatant levels of IFN γ at 48 h in cultures of mononuclear cells from umbilical cords (n=9), children (n=44) and adults (n=20). Upper panel: no stimulation. Lower panel: stimulation with pokeweed mitogen (PWM). Results are medians and quartiles. Blank box: cord blood; hatched box: children; filled box: adults. *** p<0.0005.

The supernatant levels of IFN γ in stimulated cultures correlated with the expression of CD2 ($r=0.245$, $p<0.05$), LFA-1 ($r=0.276$, $p<0.05$), CD45RO ($r=0.279$, $p<0.05$) and CD8 ($r=0.401$, $p<0.001$) (Fig. 5). IFN γ did not correlate with the expression of CD4.

Discussion

We have studied the production of IL-6, TNF α and IFN γ by MNC from umbilical cords, children and adults. The levels of IL-6 and TNF α were found to be significantly higher in children than in neonates and adults under suboptimal stimulation conditions. This indicates that the rate of production/release of

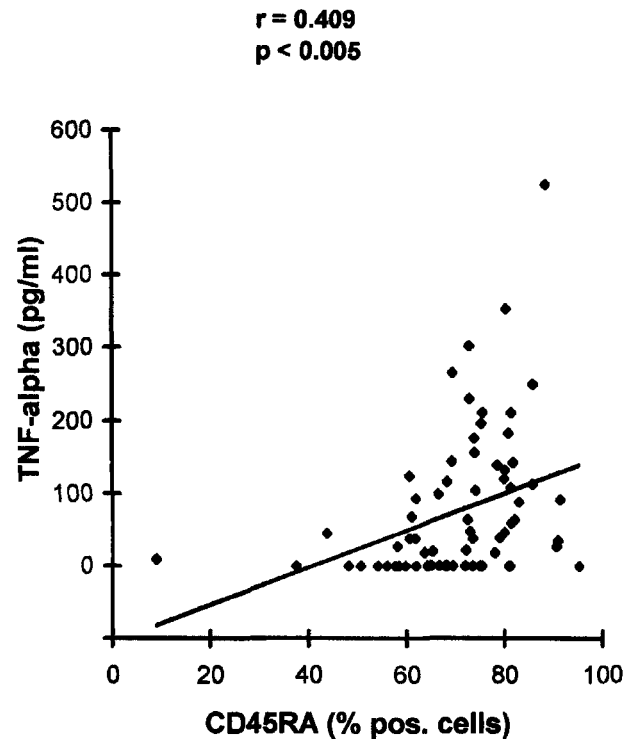


Fig. 4. Correlation between CD45RA expression and TNF α production and by MNC at 48 h in PWM stimulated cultures.

IL-6 and TNF α is higher in children than in neonates and adults under these experimental conditions, suggesting that the activation threshold of MNC with respect to release of IL-6 and TNF α is lower in children than in neonates and adults. Since both IL-6 and TNF α are important mediators of fever (17), a lowered threshold for release of these cytokines in children is in accordance with the tendency of children to react with fever more easily than adults and, in particular, newborns (18). Moreover, a relatively high threshold for IL-6 production in cord blood MNC is in line the fact that neonates seem to be less prone to mount an acute phase response during septicaemia (19).

Using optimal *in vitro* stimulation we found comparable levels of IL-6 and TNF α in the three age groups, indicating that the capacity of IL-6 and TNF α production by MNC does not vary with age. Therefore, it is not very likely that age dependent variations in the susceptibility to disseminated bacterial and viral infections are due to differences in production of IL-6 and TNF α .

Previous studies on age variation in the production of IL-1, TNF α and IL-6 have led to diverging results, with findings of comparable (10, 11) as well as reduced production in neonates and adults (12, 13). The fact that the former studies used LPS and the latter used PHA or PWM as stimulators would suggest that the contrasting results were due to the different stimulation protocols applied (LPS prima-

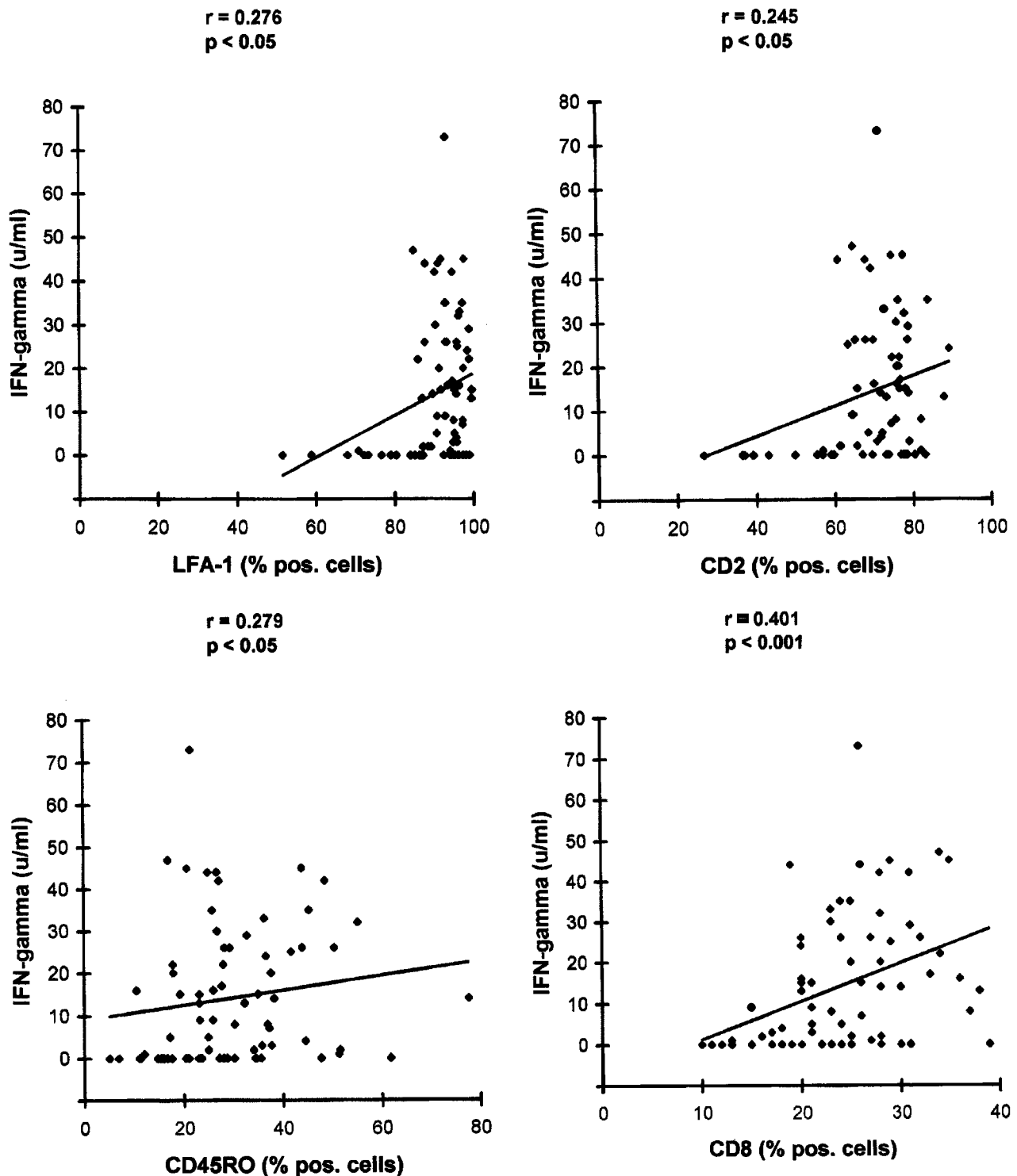


Fig. 5. Correlations between IFN γ production in PWM stimulated cultures and phenotype expression by MNC. A: LFA-1 versus IFN γ B: CD2 versus IFN γ at 48 without stimulation. C: IFN γ versus CD45r0. D: IFN γ versus CD8.

rily activating monocytes in contrast to PHA and PWM). Since we found comparable levels of IL-6 and TNF α irrespective of the stimulation procedure applied this explanation does probably not account for the diverging results reported.

Age-dependent variations in cytokine profiles in whole blood cultures from children and adults have recently been reported (14). The production of TNF alpha and IL-1 was found to be lower in children than in adults when the data were normalized with

respect to the mononuclear cell counts. The apparent discrepancy between these findings and our results is most likely due to differences in the culture systems studied. Thus, cytokine production in whole blood cultures is not dependent upon the number and the functional status of the mononuclear cells alone, but it is also influenced by the presence of the other types of leukocytes (eg. granulocytes) as well as various soluble factors present in plasma, including hormones and immunoglobulins, the level of which are known to vary with age.

In agreement with previous studies we found undetectable levels of IFN γ in neonates (5, 7, 8, 20), whereas the IFN γ levels were comparable in children and adults. IFN γ has antiviral activity and is a potent inducer of monocyte activation and expression of MHC II (4). Moreover, it plays a major role in the regulation of the Th1/Th2 balance by promoting differentiation of Th precursors into Th1 cells, both *in vitro* and *in vivo* (21). Therefore, it is likely that the high susceptibility to disseminated infections seen in neonates may partly be ascribed to low production of IFN γ .

It has previously been demonstrated that deficient IFN γ production by neonatal T lymphocytes could be restored by coculturing the neonatal T cells with adult monocytes, suggesting a functionally immaturity of neonatal monocytes (7). The IFN γ deficiency was, however, not restored by costimulating the neonatal lymphocytes with IL-1, and, therefore, not likely due to insufficient activity of this costimulatory cytokine (20). In the present study a positive correlation was noticed between IFN γ production and TNF α (in unstimulated and PWM stimulated cultures) and IL-6 levels (in unstimulated 48 h cultures), suggesting that insufficient production of IFN γ in neonates may relate to the production of IL-6 and TNF α . However, since these correlation coefficients were rather weak, and since the general pattern of TNF α and IL-6 production was different from that of IFN γ is not likely that these relations between the cytokines are of major importance.

To get further insights into the mechanisms behind the age related differences in immune responses and cytokine production the phenotypic distribution of the MNC was investigated. In agreement with previous studies the expression of CD3 and CD8 were relatively low in cord blood MNC, while the expression of the "memory cell" phenotype, CD45R0 correlated positively with age (22–26).

Cytokines production profiles are dependent upon the phenotypes of the MNC involved. In the present study it seemed, however, little likely that the observed differences in IL-6 and TNF α production profiles were due to differences in the distribution of the major MNC subsets (T cells, B cells, monocytes), because no correlation between the fre-

quencies of these cell subsets and cytokine profiles were observed.

The levels of TNF α correlated positively with CD45RA suggesting that the production of TNF α is influenced by the differentional status of the lymphocytes. However, neither TNF α nor IL-6 correlated significantly with CD45R0, CD25 or HLA-DR and therefore differences in the expression of these markers of activation and differentiation does not seem to be of major importance for the observed variations in IL-6 and TNF α .

Although low production of IFN γ has been reported in a number of previous studies the mechanism behind this deficiency remains poorly understood (5, 7–9). It has been suggested that the decreased production of IFN γ by neonatal cells is due to differences in their intrinsic capacities to produce IFN γ as well as to differences in regulatory mechanisms (8, 9, 20). The pairs of adhesion receptors, LFA-1/ICAM-1 and CD2/LFA-3, are thought to contribute to the immune activation by mediating adhesion as well as signalling (15). We observed a negative correlation of ICAM-1 with age and positive correlations between expression of CD2, LFA-1 and LFA-3 with age. Furthermore, LFA-1, CD2 and CD45R0 correlated positively with IFN γ levels. This result is in accordance with the previous finding that the CD2 and LFA-1 receptors are highly expressed on CD45R0+ T cells, a subset known to have a high capacity of IFN γ production (27). Thus, our data suggest that the low capacity of IFN γ production in neonates may partly be caused by low expression of CD2 and LFA-1 in unprimed lymphocytes of the CD45R0 phenotype.

The IFN γ levels were also found to correlate positively with CD8, which was expressed at a low degree in neonates. This is in line with the fact that CD8+ T cells are important producers of IFN γ (28). Our finding is noteworthy in the light of the fact that CD8+ T cells may play a central role in the regulation of Th1/Th2 differentiation - and B cell maturation (28).

In conclusion, we have found age related differences in the production profiles of IFN γ , IL-6 and TNF α by *in vitro* cultured MNC from neonates, children and adults. These differences may to some extent relate to differences in the expression of cell surface molecules involved in cellular interactions and signalling.

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