

CD30 on stimulated CD4⁺ T lymphocytes in newborns regarding atopic heredity

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CD30 was initially described as Ki-1 Ag on Reed-Sternberg cells of Hodgkin's lymphoma and its and CD30L⁺ expression on T cells in placenta were equally frequent in the atopic and non-atopic women. In this article we present a study of CD30 mean fluorescence intensity (MFI) on CB T CD4⁺ cells. We tested the hypothesis that in newborns with atopy family history there is a changed CB T cells response after antigen stimulation comparing with those without atopy family history. The study population consisted of 31 newborn babies (29-breastfed, two non-breastfed) and their mothers. Eleven of them had positive and 20 had negative atopy family history. Performed tests included cord blood, which was a subject to flowcytometry analysis and was cultured for 24 h, cytokine production was measured (IFN- γ , IL-4 and IL-12). Secondly, we measured total maternal and cord blood IgE levels. We studied CD30 MFI as in our studies in larger group of newborns, CD30 expression on CD4⁺ T cells appeared to be very low. MFI of CD4⁺ CD30⁺ after PHA-stimulation (213.55: range: 41.77–434.51) was significantly increased compared to MFI of CD4⁺ CD30⁺ before PHA-stimulation (43.63: range 28.67–134.67)($p \leq 0.001$). Newborns with and without atopy family history were analyzed. We found no difference between MFI of CD4⁺ CD30⁺ on non-stimulated T cells in non-atopic (43.80: range 28.66–134.66) and atopic (43.30: range 29.12–80.92) ($p > 0.05$). After PHA stimulation MFI of CD4⁺ CD30⁺ in non-atopic (273.05 (range: 42.9–434.51) was significantly increased compared with the atopic newborns to MFI of 87.1 (range: 41.78–241.42) ($p = 0.00$). We have not found any correlation between MFI of CD4⁺ CD30⁺ and total maternal and total CB IgE levels. The role of CD30 in immunological response needs further research studies.

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Atopic allergy begins usually in early life, which suggests that a child is exposed to factors promoting atopy as a neonate or during fetal life. T-cell activation is a key point for allergic response. CD30 is one of T-cell costimulatory molecules expressed on activated Th2 cells.

CD30 was initially described as Ki-1 Ag on Reed-Sternberg cells of Hodgkin's lymphoma (1). Del Prete et al. found CD30 molecule to be preferentially expressed on T cells $\alpha\beta$ Th0/Th2 (2). They suggested that Th1 clones expressed poor or no mRNA, and showed low or undetectable expression of both membrane and

soluble CD30 (sCD30) protein, whereas Th2 clones showed both CD30 mRNA and membrane CD30. Detection of CD4⁺ CD30⁺ T cells in allergic subjects may reflect the occurrence of an ongoing response to allergen stimulation. Ellis et al. (3) and Alzona et al. (4) proved CD30 expression to be linked with IFN- γ production and inhibitable by IL-4. However, Bengtsson et al. (5) showed the T cells expressing CD30⁺ were not associated with any cytokine profile.

Ekstrom et al. were investigating CD30 expression in placenta (6). CD30, CD30L⁺ expression on T cells, detected in placenta, were

equally frequent in the atopic and non-atopic group. However, sCD30 in cord blood correlated with maternal levels of sCD30.

CD45 isoforms show no phenotypic variations between newborns and adults in their respective *in vitro* responses to antigenic stimuli (7). Transformation to and acquisition of CD45RO⁺ phenotypes, particularly in the CD4⁺ subpopulation, are immunologically crucial e.g. for high IFN- γ production capability (8).

In this article, we present a study of CD30 mean fluorescence intensity (MFI) as in our studies in larger group of newborns (data not published) CD30 expression on CD4⁺ CD45RA⁺ and CD4⁺ CD45RO⁺ appeared to be very low.

We tested the hypothesis that individuals with atopy family history develop different response by T cells in cord blood after antigen stimulation compared with those without atopy family background.

Subjects and methods

The study population consisted of 31 (29-breastfed, two non-breastfed) newborns and their mothers. All the infants were born at term and had no chronic diseases or infections at the time of the study. The children were classified as having a positive ($n = 11$) or negative family history of atopic disease ($n = 20$). The degree of risk of allergy was scored according to the family history of allergy (9). Three points were given if atopy was present in mother or father or siblings. Two points were given if atopy was present in uncle or aunt or grandparents.

Blood samples

Venous blood samples (cord blood) were obtained at birth from neonates. Collection was performed by inserting a 19-gauge needle into placental vein, after careful swabbing with alcohol to remove all contaminating maternal blood. Venous blood samples from mothers were taken after delivery. The mothers gave written informed consent to participate in the study, which was approved by the ethics committee of the Medical University of Silesia, Katowice, Poland.

Flowcytometric analysis of lymphocytes

Cord blood had either flow cytometric analysis performed or it was cultured. Whole blood (2 ml) sample was placed in tube containing EDTA (Monovette; Sarstedt, Numbrecht, Germany)

and processed within 6 h of collection. Cells were incubated for 30 min with surface antigen-specific (CD3, CD4, CD45RA, CD45RO, CD30) fluorescence-conjugated [fluorescein isothiocyanate (FITC), phycoerythrin (PE) or PerCP monoclonal antibodies (mAbs)] (Becton- Dickinson, San Jose, CA, USA). Red blood cell lysis was conducted by adding 2 ml of 1 $\times$ FACS lysing solution (BD). Afterwards, cells were washed twice with PBS (phosphate- buffered saline) and acquisition of the data was processed by FACScan (BD). Ten-thousand cells were acquired and data were analyzed with CELLQUEST (BD) software. Isotype controls were included as appropriate.

Mononuclear cells were prepared from heparinized umbilical cord blood by gradient-centrifugation at 700 g over Histopaque (Sigma, St Louis, MO, USA). Cells at the interface were collected and washed three times with PBS and resuspended in RPMI supplemented with 10% fetal calf serum, 50 μ M β -mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin sulfate. Cells were cultured and stimulated with 10 μ g/ml of phytohemagglutinin (PHA). After activation, the cells were stained with surface antigen-specific (CD3, CD4, CD45RA, CD45RO, CD30) fluorescence-conjugated FITC, PE or PerCP mAbs (BD) and were processed for flowcytometric analysis as described above.

Cytokine measurement

For cytokine measurement 10 ml of peripheral blood was taken and the samples were processed within 6 h. IFN- γ , IL-4 and IL-12 were measured using HS Quantikine kit (R&D Systems, Minneapolis, MN, USA). The samples were diluted twice with PBS and PBLs and then isolated using Histopaque and centrifugation (700 $\times g$). Human peripheral blood mononuclear cells (1×10^6 cells/ml) were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 50 μ M β -mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml of streptomycin sulfate. Cells were cultured for 24 h in a humidified 5% CO₂ atmosphere at 37°C.

IgE level assessment

Plasma samples were stored at -20°C until processed for IgE measurements (both maternal and cord blood). Serum IgE levels were determined by fluoroenzymeimmunoassay using UniCAP kits (Phadia, Uppsala, Sweden). The

sensitivity for maternal total IgE was 2 IU/ml according to manufacturer's instructions. Cord blood IgE levels were determined on a low-level fluoroenzymeimmunoassay from Pharmacia with a sensitivity of <0.35 IU/ml.

Statistical analysis

Significance of difference was determined by the Mann-Whitney *U*-test and Wilcoxon's Rank Sum test for comparison of groups. The correlation coefficient was used as a measure of co-variation between two variables. Data were analyzed using MEDCALC 7.0 (Mariakerke, Belgium) and STATISTICA 6.0 (Krakow, Poland).

Results

The expression of numerous markers by CD4⁺ T cells was examined. CD4⁺ T cells were identified by characteristic side scatter and CD4 positivity.

The geometric mean range for MFI of CD4⁺ CD30⁺ in all newborns was 43.63 (range: 28.67–134.67) before stimulation, 213.55 (range: 41.77–434.51) after stimulation and delta (difference after-before cells were stimulated) was 169.91 ($p \leq 0.001$)(Fig. 1).

The group was divided according to atopic heredity. The geometric mean range for MFI of CD4⁺ CD30⁺ found in newborns with negative family history was: 43.80 (range: 28.66–134.66) before stimulation, 273.05 (range: 42.9–434.51) after stimulation and delta-229.27. Comparably the geometric mean range for MFI of CD4⁺ CD30⁺ found in newborns with positive family history was: 43.30 (range: 29.12–80.92) before stimulation, 87.1 (range: 41.78–241.42) after stimulation and delta-43.80. Fig. 2 shows

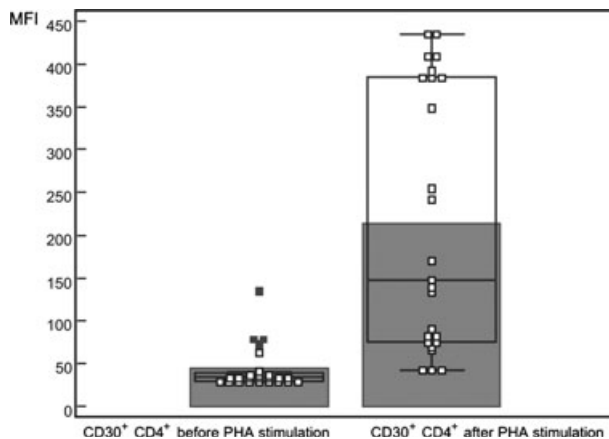


Fig. 1. Mean fluorescence intensity of CD30⁺ CD4⁺ before and after T cells stimulation.

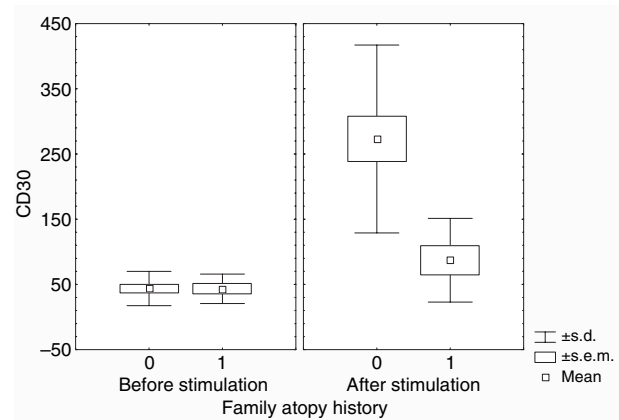


Fig. 2. Mean fluorescence intensity of CD30⁺ CD4⁺ on CB T cells of non-stimulated and stimulated T cells in atopic and non-atopic newborns.

MFI of CD30⁺ CD4⁺ expression before and after T cells were stimulated regarding atopic heredity. The difference between MFI of CD30⁺ CD4⁺ in the two groups (positive vs. negative family atopy history) before stimulation was not significant; however, statistically significant difference was observed between the two groups after T cells stimulation ($p < 0.05$).

Further, we analyzed the correlation between maternal total IgE and MFI of CD4⁺ CD30⁺ in the whole group. The correlation between maternal total IgE and MFI of CD4⁺ CD30⁺ before and after T cells stimulation was statistically not significant ($p > 0.05$), which is shown in Table 1. Similarly, there was no correlation between CBIgE and MFI of CD4⁺ CD30⁺ ($p > 0.05$).

Finally, we studied the correlation between IFN- γ , IL-4 and IL-12 produced by stimulated mononuclear cells and MFI of CD4⁺ CD30⁺ of stimulated cells. The Spearman correlation test showed results as presented in Table 2.

Discussion

Discussion about the importance of CD30 molecule in allergy pathogenesis is still ongoing and

Table 1. Correlation between mean fluorescence intensity (MFI) of CD30⁺ CD4⁺ and maternal/cord blood total IgE

	Spearman <i>R</i>	$\sqrt{N-2}$	p-level
MFI of CD30 ⁺ CD4 ⁺ and maternal total IgE	0.089	0.429	0.67
MFI of CD30 ⁺ CD4 ⁺ after cells stimulation and maternal total IgE	-0.185	-0.905	0.37
MFI of CD30 ⁺ CD4 ⁺ and cord blood total IgE	-0.066	-0.320	0.75
MFI of CD30 ⁺ CD4 ⁺ after cells stimulation and cord blood total IgE	-0.825	-1.426	0.17

Table 2. Mean fluorescence intensity (MFI) of CD4⁺ CD30⁺ of stimulated cells correlated with IFN- γ , IL-4 and IL-12 produced after *in vitro* stimulation with phytohemagglutinin and ovalbumin

	Spearman <i>R</i>	t(N-2)	p-level
MFI of CD30 ⁺ CD4 ⁺ and IFN- γ level before stimulation	0.163	0.739	0.46
MFI of CD30 ⁺ CD4 ⁺ and IFN- γ level after PHA-stimulated	-0.631	-3.639	0.00
MFI of CD30 ⁺ CD4 ⁺ and IFN- γ level after OVA-stimulated	0.107	0.484	0.63
MFI of CD30 ⁺ CD4 ⁺ and IL-12 level before stimulation	-0.479	-2.502	0.02
MFI of CD30 ⁺ CD4 ⁺ and IL-12 level after PHA-stimulated	-0.076	-0.353	0.72
MFI of CD30 ⁺ CD4 ⁺ and IL-12 level after OVA-stimulated	-0.180	-0.840	0.40
MFI of CD30 ⁺ CD4 ⁺ and IL-4 level before stimulation	-0.183	-0.854	0.40
MFI of CD30 ⁺ CD4 ⁺ and IL-4 level after PHA-stimulated	0.078	0.361	0.72
MFI of CD30 ⁺ CD4 ⁺ and IL-4 level after OVA-stimulated	-0.288	-1.381	0.18

research studies have shown that CD30 is linked with atopy diseases such as atopic dermatitis in children (10, 11). On the other hand, it was shown that sCD30 in cord blood was not related to atopy in childhood (12). Because of still undetermined role of CD30, especially in newborn's immunological system, we examined the expression of CD30 on CD4⁺ T cells in cord blood of healthy newborns born at term without any signs of infection. In parallel, we measured maternal total IgE and CBIgE to evaluate whether CD30 expression on T cells is changed in children with positive family atopy history.

We found an increase in MFI of CD30⁺ CD4⁺ after stimulation, which is consistent with the study by Chipeta (7) where CD45RO isoforms in newborns and adults respond equally to antigenic stimuli in *in vitro* studies. Our data showed that CD30 upon stimulation is greatly expressed on T cells and this marker is thought to be present on activated T cells. We observed a sixfold elevation of MFI of CD30⁺ CD4⁺ before and after stimulation. The increase in CD30⁺ CD4⁺ T cells if PHA-stimulated, suggests that neonatal T cells are potent to respond to antigens and possibly be an integral part of immunological response.

In the present study, we found that MFI of CD30⁺ CD4⁺ of T cells upon stimulation was three times higher in the group with no atopy family history than MFI of CD30⁺ CD4⁺ in the group with positive family atopy history. T-cell activation is a key point for allergic response. However, the precise mechanisms of its activation are yet unclear. CD30 is known as an activation marker belonging to the TNFR superfamily (13) that may be involved in a sequence of costimulatory mechanisms. Thus, the binding of CD30 to the CD30 ligand (CD30/CD153) is believed to be important in several immunoregulatory processes (14) and the expression of this receptor is depending on cell type and nature of microenvironment (15). One of the

explanations that MFI of CD30⁺ CD4⁺ was lower in positive atopy history may be that the allergen stimulation at pregnancy in newborns with positive atopy family history was able to satiate T cells and the dose of mitogen required to stimulate cells in *in vitro* studies must have been higher than previously thought. There is also a possibility that atopic surroundings interact inhibiting CD30⁺ CD4⁺ T cells. The previous idea (15) that CD30 expression is driven by allergic microenvironment may not be appropriate to adjust in our study.

Another possible hypothesis proposes CD30 molecule to be possibly linked with Th1 type of immunological response. This might have explained a difference in MFI of CD30 after stimulation between group of newborns with or without atopic family history. CD30 might follow in its function another molecule expressed on memory T cells a CD45RO, which is important for high IFN- γ production capability (8). In our study, a negative correlation between produced IFN- γ and MFI of CD30⁺ CD4⁺ after PHA-stimulation was shown. Interleukin-4 did not correlate with MFI of CD30⁺ CD4⁺. In another study by Bengtsson (5) no linkage between T cells expressing CD30⁺ and secreted cytokines was found. However, these results might be limited by the fact that the capacity to produce cytokines, especially IFN- γ or IL-4 is physiologically low in newborn infants (16, 17).

An increased production of IL-4 and a decreased production of IFN- γ in atopic diseases has been demonstrated in many studies (16, 18) so the decline in MFI of CD30⁺ CD4⁺ after PHA-stimulation with rising levels of IFN- γ may indicate that CD30 is associated with Th-2 type response as it was previously suggested (19). Contradictory to other papers, it was also shown that CD30 crosslinking enhances the cytokine (IL-4, IFN- γ) production in both Th1 and Th2 cells (20), but research studies on CD30 are hardly comparable mainly due to different

sample selections. Our demonstration showed interleukin-12 is negatively correlated with MFI of CD30⁺ CD4⁺ before stimulation, which indicates also that CD30 may probably be associated with Th2 cells. IL-12 promotes Th1 differentiation, enhances production of Th1 cytokines such as IL-2 and IFN- γ (21) and inhibits Th2 cytokine synthesis (22). Our observation on increasing levels of IL-12 with decreasing MFI of CD30⁺ CD4⁺ may suggest that Th2- directed T cells express CD30. Del Prete (19) showed that CD30 is preferentially expressed on T cells $\alpha\beta$ Th0/Th2 and Th2 clones present both CD30 mRNA and membrane CD30. He suggested the detection of CD4⁺ CD30⁺ T cells in allergic subjects may reflect the occurrence of an ongoing response to allergen stimulation which in recent years is still under discussion. However, Ekstrom found no difference existing between CD30, CD30L⁺ cells, detected in placenta, and sCD30 levels in correlation with maternal atopic heredity (6).

In this investigation, similar to the study by Ekstrom (6), we did not confirm any correlation between MFI of CD30⁺ CD4⁺ after stimulation and CB and maternal IgE levels. The new finding of difference between MFI of CD30⁺ CD4⁺ in newborns with atopic and non-atopic family history directed our study to examine whether maternal or cord blood total IgE is correlated with MFI of CD30⁺ CD4⁺. In this investigation, similar to the study by Ekstrom (6), we found no association between the two co-variables. There is a possibility that IgE antibodies can be also involved in a response to parasite infection, but in our geographical lieu is hardly possible. The recent study by Heshmat showed that sCD30 did not correlate with age or serum total IgE, on the other hand it correlated with the severity of AD and bronchial asthma (23). The putative role in allergies for CD30 molecule and CBIgE is still not confirmed. Neither sCD30, sCD23 nor sIL-4R was found to be sufficiently good in allergy prediction during infancy or early childhood (24, 25). At the same time, the cut-off values for CBIgE levels are under discussion. The lowest suggested CBIgE level as predictor for atopic disorders development was 0.3 kU/l but it appeared to present poor specificity. In another study by Kjellman et al., newborns with CBIgE higher than or equal to 0.9 kU/l developed atopic disease by 6 yr of age in 80% of cases (26). The correlation between MFI of CD30 and CBIgE was not significant but it need further investigation in a larger group as a possible way to understand immunological mechanisms appearing in newborns.

An obvious limitation of the study is a relatively small sample. However, it must not be forgotten that in healthy donors only few activated mononuclear cells in lymphoid tissue show CD30 expression.

CD30 molecule and its function need further investigation but certainly it has an important role in immunological response.

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