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# Costimulatory molecules expression and cytokine profiles of cord blood mononuclear cells in newborns with low and high risk of developing atopic diseases

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This study sought to determine predictors of atopic diseases in newborns. We evaluated the levels of expression of costimulatory molecules (CD80 and CD86) and the production of cytokines [interleukin (IL)-12, interferon (IFN)-γ, IL-4, IL-10] in the cord blood of mononuclear cells in high risk newborns (n = 17), and compared them with those in low risk newborns (n = 25). Fluorescence-activated cell sorter (FACScan) analysis was performed to determine the expressions of CD80 and CD86 on activated B cells and monocytes of both groups. The levels of IL-10, IL-12p40 and IL-12p70 in the supernatant were assayed by enzyme-linked immunosorbent assay (ELISA), and also the mRNA levels by reverse transcription-polymerase chain reaction (RT-PCR). Intracellular staining of IL-4 and IFN-γ in stimulated mononuclear cells was also performed as well. The expressions of CD80 and CD86 on B cells showed no significant difference between the high and low risk group. There was greater expression of CD86 on the monocytes of low risk newborns as compared to high risk newborns ($p < 0.05$). When B cells and monocytes isolated from the cord blood of both groups were stimulated with mitogens, the production of IL-10, IL-12p40, and IL-12p70 in the supernatants was not significantly different. The expressions of mRNA of IL-10, IL-12p35, and IL-12p40, and the intracellular staining of IL-4 and IFN-γ in stimulated mononuclear cells were not significantly different between the two groups. These findings suggested that cytokine profiles in the cord blood cannot predict the development of atopic diseases. Determination of whether preferential expression of costimulatory molecules is of predictive value or not will require further study.

**Key words:** Atopic diseases, cord blood, costimulatory molecules, cytokines

The allergen-specific T cells in atopic patients are known to be biased to the production of type 2 cytokines in contrast to the type 1 cytokines in normal individuals [1-3]. The differentiation of precursors of helper T (Th) cells is determined by many factors, among which, the costimulatory factors on antigen-presenting cells (APCs) and cytokine profiles have been suggested to play important roles [4,5].

T cells activation requires at least two signals. One signal is delivered by the peptide-major histocompatibility complex (MHC). The second, costimulatory signal is provided by binding of a signal-transmitting protein on T cell surface with a specific ligand on APCs. One of the best characterized pathways is that mediated by the CD28 on the T cells and its receptor CD80 (B7-1) and CD86 (B7-2) on the APCs [1-2]. Treatment targeting at this costimulation pathway has shown promise in animal models of autoimmune diseases and graft rejection [6-9].

It has been observed that CD28/CD80/CD86 plays an important role in the Th2 response and the productions of interleukin 4 (IL-4) and interleukin 5 (IL-5) [10]. The cytokines including IL-12, interferon (IFN)-γ, IL-4, IL-10 have also been shown to modulate the differentiation of naive Th cells [3-5].

Long-term observations have found that children are prone to develop allergic diseases when their family members have a history of atopy [11]. The clinical symptoms and signs of atopic diseases such as itching skin rashes, cough, wheezing, and rhinorrhea usually do not occur at birth, and some develop even as late as in adulthood [12]. The aim of this study was to determine the possible factors predictive of atopy at birth by examining the levels of the costimulatory molecules (CD80 and CD86) on the cord blood APCs and the cytokines producing by these cells in high risk

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newborns, and to compare these levels with those in low risk newborns.

## Materials and Methods

### Study population

During the period from February 1997 to May 1997, 17 newborns with high risk of allergy and 25 newborns with low risk of allergy were recruited for participation at the Department of Obstetrics and Gynecology of National Taiwan University Hospital. The risk of developing of allergic disease was rated according to a family allergy score (FAS) (Table 1). Newborns with an FAS of no less than 3 were defined as high risk group and those with an FAS of less than 3 were defined as low risk.

### Cord blood and separation of mononuclear cells

Cord blood was obtained at the delivery room of the National Taiwan University Hospital. The mononuclear cells were separated by Ficoll-Paque density gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden), washed in 1 x Hank's buffered saline solution, and then resuspended in RPMI-1640, supplemented with 10% heat-inactivated fetal calf serum, 100 μg/mL streptomycin, 100 IU/mL penicillin, and 0.25 μg/mL amphotericin B.

### Enrichment of B cells and monocytes and cell cultures

Monocytes were recovered from mononuclear cell suspension by adherence to tissue culture flask (25T) for 3 days at 37 °C in 5% $CO_2$. Non-adherent cells were removed from the monocyte monolayers by washing with warm (37 °C) PMI-1640 medium. Monocytes were detached by treatment with ice-cold phosphate-buffered saline without calcium and magnesium supplemented with 0.02% ethylenaminetetraacetic acid (EDTA; Sigma, Deisenhofen, Germany) and gentle scraping with a cell lifter (Costar, Cambridge, MA, USA). B cells were purified from mononuclear cell suspension by treatment with anti-CD19 and magnetic bead separation as previously described [13].

**Table 1.** Family allergy score

| Category | Score A | Score B |
|---|---|---|
| Father | 3 | 2 |
| Mother | 3 | 2 |
| Siblings | 3 | 2 |
| Grandparents | 2 | 1 |
| Uncles and Aunts | 2 | 1 |

Score A: one subject has the diagnosis of atopic dermatitis, atopic asthma, or allergic rhinitis and with active symptoms of these allergic diseases in recent 1 year.
Score B: one subject has been once diagnosed with atopic dermatitis, atopic asthma, or allergic rhinitis but without active symptoms for more than 1 year.

### Flow cytometric analysis cell surface marker and costimulator expression

The cell suspension was washed with phosphate-buffered-saline (PBS) supplemented with 0.05% $NaN_3$ before incubating with florescence-labeled monoclonal antibodies (mAb) (anti-CD45 mAb, anti-CD14 mAb, anti-CD3 mAb, anti-CD19 mAb, anti-CD80 mAb and anti-CD86 mAb) on ice for 30 min. After washing and resuspension in buffer fluid, the cells were analyzed with a fluorescence-activated cell sorter (FACScan) (Becton Dickinson, Mountain View, CA, USA). B cells were determined by gating on CD19 positive cells, whereas monocytes were gated on CD14 positive cells.

### Determination of cytokine production by ELISA and RT-PCR

B cells and monocytes were cultured in RPMI-1640 medium, supplemented with 10% heat-inactivated fetal calf serum, 100 μg/mL streptomycin, 100 IU/mL penicillin, and 0.25 μg/mL amphotericin B. After stimulation by *Staphylococcus aureus* Cowan I strain (SAC) (1:50,000) and lipopolysaccharide (LPS) (5 μg/mL), respectively, in 37 °C for 48 h, the supernatant was harvested and stored at -70 °C for later enzyme-linked immunosorbent assay (ELISA) (IL-10, IL-12p70, IL-12p40). Total cellular ribonucleic acid (RNA) was extracted by TRIZOL (Gibco Life Technologies). First strand cDNA was synthesized at 42 °C for 1 h using 1 μg RNA in $dH_2O$, 4 μL 5 x RT buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 50 mM dithiothreitol, 15 mM $MgCl_2$), 0.5 μL (20 U) RNAs in (Promega, WI, USA), 1 μL dNTPs mixture, 2 μL oligo-dT primer and 200 units reverse transcriptase (Gibco Life Technologies). First strand cDNA samples were stored at -20 °C until further use. Polymerase chain reaction (PCR) was conducted using primers purchased from Clontech (Palo Alto, CA, USA). Primers sequences used include IL-10 (5' primer, AAGCTCAGAACCAAGACCCAGACATCAA-GGCG; 3' primer, AGCTATCCCAGAGCCCCA-GATCCGATTTTGG), IL-12 p35 (5' primer, GAGAGAGACACAGAAGGAGA; 3' primer, GATTACCCTCAACGGACCGGAG), IL-12 p40 (5' primer, GCAACATGTGTCACCAGCAGTT; 3' primer, AAGACCTGCAAAGTGGACGACC) and G3PDH (5' primer, TGAAGGTCGGAGTCAACGGATTTGGT; 3'

primer, CATGTGGGCCATGAGGTCCACCAC). Ten μL of cDNA were amplified in reaction tube with 1 μL of 5' primer and 3' primer, 10 μM dNTPs, reaction buffer, two units SuperTaq polymerase (ProTech), 25 μM $MgCl_2$ and DEPC-$H_2O$ in a final volume of 50 μL. The reaction mixtures were overlaid with a drop of light mineral oil, and PCR was performed in a DNA thermal cycler (Perkin Elemer Cetus Corp.) for 35 cycles: 45-sec denaturation at 94 °C, 45-sec annealing at 60 °C, and 2-min extension at 72 °C. The PCR product was visualized by subjecting it to the electrophoresis with 10 μL of reaction mixture on ethidium bromide stained 2% agarose gel.

### Intracellular staining of IL-4 and interferon-γ

The mononuclear cell were stimulated with PMA (20 ng/mL) + ionomycin (1 μM) in the presence of monensin (3 μM) for 12 h. The cells were then spun down and washed twice in staining buffer (Dulbecco's PBS containing 5% heat-inactivated fetal bovine serum and 0.1% sodium azide). Surface staining was done with anti-CD4 mAb at 4 °C for 30 min. After washing with PBS, fixation buffer (Dulbecco's PBS containing 4% w/v paraformaldehyde) was added for 20 min. Intracellular staining with anti-IFN-γ anti-IL-4 mAb was done at 4 °C for 10 min after permeabilization. After washing twice, the samples were fixed with formaldehyde and analyzed by FACScan.

## Results

### Enrichment of B cells and monocytes

After retention by immunomagnetic beads coated with anti-CD19 mAb and isolated from mononuclear cells by culture in 25T flask, respectively, the purity of B cells checked by flow cytometric analysis of $CD19^+$ population and the purity of monocytes by $CD14^+$ population was near 80%.

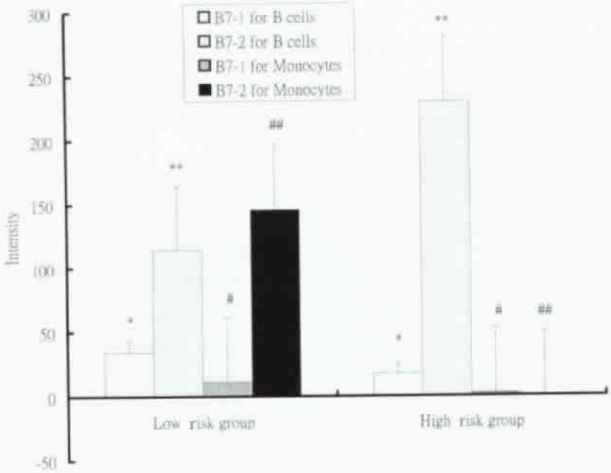


**Fig.1.** Expressions of CD80 (B7-1) and CD86 (B7-2) on B cells and monocytes of cord blood between low and high risk newborns. (*$p > 0.05$, ** $p > 0.05$, # $p > 0.05$, ## $p < 0.05$)

### Expression of CD80 (B7-1) and CD86 (B7-2) after stimulation

The costimulatory molecule expression on B cells and monocytes is displayed in Fig. 1. There was no significant difference in the expression CD80 and CD86 on B cells between the two risk groups. On monocytes, the samples from low risk subjects showed a higher level of CD86 as compared to those from high risk subject while there was no significant difference in the expression of CD80 between the two groups.

### Production of cytokines by cord blood B cells and monocytes

#### *Interleukin-10*

In B cells, the production of IL-10 by samples from the low risk group samples was undetectable by ELISA (Table 2). Only one sample in the high risk group had detectable IL-10 (21.18 pg/mL) in the supernatant. In monocytes, there was no significant difference in the

**Table 2.** The level of IL-10, IL-12p40, and IL-12p70 produced by B cells and monocytes in newborns with low (n = 25) and high risk (n = 17) of atopy by ELISA method

| | Low risk group | | High risk group | |
|---|---|---|---|---|
| | B cells | Monocytes | B cells | Monocytes |
| IL-10 (pg/mL) | ND | 3451 ±2027[a] | 21.2[d] | 2640 ±1943[a] |
| IL-12p40 (pg/mL) | 482 ±221[b] | 496 ±39[c] | 568 ±535[b] | 568 ±483[c] |
| IL-12p70 (pg/mL) | 29[e] | ND | 29[f] | ND |

[a] $p > 0.05$.
[b] $p > 0.05$.
[c] $p > 0.05$.
[d] Only one (1/17) had detectable IL-10 with the level of 21.2 pg/mL.
[e] Only one had detectable (1/25) IL-12p70 with the level of 29 pg/mL.
[f] Only one (1/17) had detectable IL-12p70 with the level of 29 pg/mL.

expression of IL-10 between the two groups (low risk: 3451 ±2070 pg/mL, high risk: 2640 ±1943 pg/mL, $p > 0.05$).

### *Interleukin-12*

In each group, there was only one sample that had a detectable IL-12 p70 level (29 pg/mL in both samples) in the supernatant of B cell culture. None of the monocytes cultures in the two groups had detectable IL-12 p70. There was no significant difference between the two groups in the level of production of IL-12 p40 by B cells (low risk: 482 ±221pg/mL, high risk: 568 ±535 pg/mL, $p > 0.05$) and by monocytes (low risk: 496 ±39 pg/mL, high risk: 568 ±483 pg/mL, $p > 0.05$).

### Expression of mRNA of cytokines (RT-PCR)

The mRNA expression of IL-10, IL-15p35, and IL-12p40 stimulated by B-cells and monocytes from high risk and low risk newborns did not show a significant difference. In B cells, there was one sample (1/25) in the low risk group and three (3/17) in the high risk group with detectable IL-10 mRNA. IL-12 p35 mRNA was detected in more samples in the low risk group (low risk 8/25 vs High risk 2/17), IL-12p40 was not detected in the B cells of either low risk or high risk newborns. In monocytes, the IL-10 mRNA was detected in three samples in the low risk group (3/25) and one in the high risk group (1/17). IL-12p35 mRNA was detected in six samples (6/25) in the low risk group and one (1/17) in the high risk group. Only one of the monocyte cultures in the high risk group had a positive reaction for IL-12p40 mRNA.

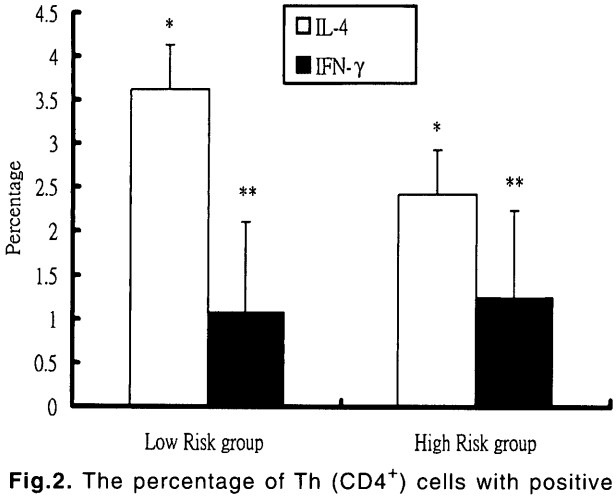


**Fig.2.** The percentage of Th ($CD4^+$) cells with positive intracellular staining for IL-4 and IFN-γ in cord mononuclear cells between newborns with low and high risk of atopy. ($^*p > 0.05$, $^{**}p > 0.05$)

### Intracellular staining of IL-4 and interferon-γ

The percentage of Th ($CD4^+$) cells with positive intracellular staining of IL-4 and IFN-γ was not significantly different between the two groups (Fig. 2).

## Discussion

Of the known etiologies of allergy, genetic and common environmental factors are the two most potent. Many epidemiological studies have revealed the significant familial aggregation of the phenotypes associated with allergic diseases [11,14]. Children with strong family histories of atopy are susceptible to atopic dermatitis, asthma, and allergic rhinoconjunctivitis. Whether prediction of atopic bias in high risk newborns can be performed by objective laboratory methods remains unclear. In this study, we evaluated the levels of costimulatory molecules on APCs and cytokines (IL-12, IFN-γ, IL-4, IL-10) in cord blood, all of which are considered to be crucial in the Th1/Th2 cells development pathways [1-5].

The results of this study showed that neonates with high risk and low risk of developing atopy, and stimulation of cord blood specimens with mitogens of SAC and LPS did not cause significantly different expression of CD80 on B cells and monocytes, or CD86 on B cells. Greater CD86 expression on monocytes was found in low risk newborns. The roles of CD80 and CD86 in the differentiation of Th naive cells into Th1 or Th2 cells have been extensively studied, with different conclusions [15]. Lenschow *et al* [6], in a study of the effects of treatment of autoimmune diabetes mice with anti-B7-1 and anti-B7-2 monoclonal antibodies, showed that blockade of CD80 costimulation accelerated the development of disease by up-regulating Th1 cells response, while anti-CD86 antibody increased the proportion of Th2 cells and prevented the disease. This *in vivo* animal study indirectly indicated that the CD80 promoted Th2 and CD86 promoted Th1 cells development. Another study reported by Burastero *et al* [16] have shown that increased expression of CD80 on alveolar macrophages, which have been involved in the amplification of allergen-specific Th2 cells. Nevertheless, other studies have failed to identify any differences between the expression of CD80 and CD86 during the development of Th1 and Th2 cells [17-18].

Other *in vivo* and *in vitro* studies have directly or indirectly demonstrated that CD80 was required for the generation of Th1 response, and that CD86 induced the development of a Th2 response in Th naive cells. For example, Kuchroo *et al* [2] have found that anti-B7-1 reduced the incidence of allergic encephalitis which was induced by autoreactive Th1 cells, and that anti-B7-2

increased disease activity. By using specific inhibitory monoclonal antibodies against CD86 and CD80, Van Neerven *et al* [19] found that CD86, rather than CD80 seemed to be the costimulatory molecule involved in the activation and proliferation of Th2 cells. In our study, the differences of the expression of CD80/CD86 on APCs between the two groups were generally not significant. This may have been because the APCs derived from cord blood were immature. Allergens and antigens stimulation have been postulated to be necessary in the expressions of costimulatory molecules on APCs [2]. Several studies have found that increased proliferation of cord blood mononuclear cells stimulated by allergens or antigens was associated with subsequent development of allergic diseases, and indicated the presence of intrauterine sensitization [20,21]. However, two recent studies have revealed that sensitization to allergens or antigens might be a common feature during pregnancy, regardless of the presence of a family history of atopy [22,23]. This finding might be an another possible reason for our results of the expressions of costimulatory molecules on cord blood APCs.

In addition to Th2 cells, many other cells such as B cells and monocytes produce IL-10 also [24,25]. In the modulation of naive Th cells differentiation, IL-10 inhibits Th1 cells development by blocking IL-2 production [26]. Our study found that the production of IL-10 by monocytes in cord blood specimens was not significantly different between neonates with low risk and high risk of atopy, and that IL-10 produced by B cells from cord blood specimens was not detectable in most newborns. IL-12 has been revealed to play a role in the priming of unstimulated Th cells into Th1 clones upon antigenic stimulation [3]. In this study, the expression of IL-12 produced by B cells and monocytes was not significantly different between cord blood specimens from high risk and low risk neonates regardless of the protein or at mRNA levels. IFN-γ and IL-4 are the major cytokines produced by Th1 and Th2 cells, respectively [5]. By means of intracellular staining and FACScan analysis, the ratio of Th1 and Th2 cells will be detectable. The proportion of Th2 cells was not significantly increased in the cord blood of high risk newborns.

In conclusion, this study failed to predict an allergic tendency at birth through detection of costimulatory molecules on APCs, IL-10 and IL-12 produced by APCs of cord blood in newborns. However, the developmental pathways of Th1/Th2 cells are complex. More stimulatory factors and cytokines, in addition to the ones enrolled in our study, are involved. The differences in the levels of these factors between neonates with high risk or low risk of atopy may become significant with progressive exposure to environmental allergens. Furthermore, the incidence of allergic diseases in children previously classified as being at a high risk of atopy remains to be clarified. Therefore, further extensive studies and long-term follow-up are necessary to determine whether laboratory analysis of cord blood may be able to predict the development of atopy.

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