B7 costimulation and intracellular indoleamine-2,3-dioxygenase (IDO) expression in umbilical cord blood and peripheral blood of healthy pregnant women

Doctoral thesis

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Introduction

It is well-known that the immune response efficiency is decreased during the perinatal period as compared to adulthood. Several factors may play a role in this finding, including immaturity of adaptive immune responses, as well as alterations in the prevalence and functionality in elements of humoral and cellular immune reactions compared to adult-type immunity. The process of antigen presentation and adequate T cell function are cornerstone features in coordinating the immune response at an early age. Over the recent decades, several studies have revealed remarkable details that contribute to these alterations. However, many aspects of the exact mechanisms are still not fully understood.

Decreased level of antigen presentation, lower expression of costimulatory molecules, lower Th1 and Th17 response, and deficient function of regulatory elements are the most important differences in CB compared with adult peripheral blood. These differences are of practical importance from two distinct aspects. First, the decreased efficiency of the immune response plays an important role in the development of several diseases affecting preterm and term neonates, as well as in a higher incidence of infections compared to adults. Second, umbilical cord blood (UCB)-derived hematopoietic stem cells are widely used in the treatment of different hematological and immunological disorders. The prevalence of graft-versus-host disease (GVHD) is lower upon UCB-derived stem cell transplantation compared to adult peripheral blood or bone marrow-derived stem cells.

Since the concept is half of foreign origins, presenting paternal antigens, it is considered a semi-allograft to maternal immunity. Therefore, a maternal immune tolerance must develop to avoid immunological rejection of the fetus. The alterations contributing to maternal tolerance are present not only at the maternal-fetal interface, but also at the systemic level. Several components of this pregnancy-

specific immune tolerance have been described over the recent years. One of the most important factors is the decreased level of activation of T cells compared to the non-pregnant state. The second and third trimesters of pregnancy are characterized by a shift of the inflammatory balance towards the anti-inflammatory direction via the upregulation of Th2 cells and a decrease in the Th17/regulatory T cell (Treg) ratio. The kinetics of calcium influx upon stimulation via the T cell receptor (TCR) is decreased in Th1 and CD8 cells compared to lymphocytes isolated from non-pregnant women. Several other factors may account for the decreased level of peripheral T lymphocyte activation in healthy pregnancy (HP). The role of the B7 family of costimulatory molecules in the development of the systemic maternal immune tolerance has not been studied before.

Antigen presentation and costimulation are the initial steps in adequate T cell function and play an important role in the coordination of downstream events in immune response. Alterations in the expression of costimulatory molecules and receptors may influence differences observed between immunological reactivity of umbilical cord blood (UCB) and adult peripheral blood (APB) T lymphocytes. B7 costimulatory molecules are expressed on antigen presenting cells (APCs) and are important regulators of T cell activation (Figure 1). Upon the engagement of the T cell receptor (TCR), the costimulatory signal from B7-1 (CD80) or B7-2 (CD86) via CD28 induces the production of IL-2 in T cells, thus protecting them from apoptosis and anergy. Both the TCR and CD28 are constitutively expressed by most naive T cells, enabling them to respond to the antigen being presented. Without costimulation, the signal from the TCR induces the tolerance of T cells to their cognate antigen instead of being activated. Nevertheless, B7 family members mediate not only stimulatory, but also inhibitory effects on T cells and therefore may contribute to the lower reactivity of UCB T lymphocytes compared to APB. Upon the stimulation of TCR, cytotoxic T lymphocyte antigen 4 (CTLA-4, CD154) becomes

phosphorylated, resulting in its stabilization on the cell surface. This way CTLA-4 can compete with CD28 for B7 binding, thus blocking the costimulatory signal and preventing IL-2 production. The affinity of the inhibitory receptor, CTLA-4 is higher than that of CD28 for B7-1 and B7-2. Besides its competitive role, CTLA-4 further emits inhibitory signals, thus contributing to the prevention of T cell activation. Another B7 family member, B7-H1 (CD274) possesses mostly, but not exclusively inhibitory properties on T cells. Its inhibitory function occurs by signalling through the programmed death-1 receptor (PD-1, CD279), inducing apoptosis or anergy of self-reactive T cells. Genetic deletion of PD-1 results in severe autoimmunity due to the loss of peripheral tolerance of self-reactive T cells. B7-H2 (CD275) serves as the ligand for inducible costimulator of T cells (ICOS, CD278), and promotes T cell activation, differentiation, and effector responses. In contrast to the costimulatory effect of CD28, ICOS most effectively induces IL-10, but does not influence IL-2 production. ICOS also stabilizes IL-10R expression on T cells, increasing their sensitivity to IL-10. Thus, the B7-H2/ICOS pathway preferentially regulates the effector function of T cells.

Therefore B7 costimulatory molecules are expressed on antigen presenting cells are important regulators of T cell activation. Besides initiating signal transduction in T lymphocytes, B7-1 and B7-2 may back-signal into the APC and influence the local immune environment through induced expression of immunosuppressive factors independently of their effects on T cells. For instance, reverse signalling through B7-1 and B7-2 after ligation by a soluble form of CTLA-4 was shown to upregulate the tryptophan (TRP) catabolic enzyme, indoleamine 2,3-dioxygenase (IDO). The potent immunosuppressive activity of IDO was first identified in pregnancy, when it was demonstrated that inhibition of IDO activity abolished allogenic gestation in mice. In the first steps of the kynurenine (KYN) pathway, TRP is transformed into KYN by IDO. KYN is then further metabolized by different enzymes. One of them is kynurenine

aminotransferase, leading to the production of kynurenic acid (KYNA), a broad-spectrum endogenous antagonist of excitatory amino acid receptors with emerging recent implications in immunomodulation. The rate of TRP degradation, represented by the kynurenine / tryptophan (KYN / TRP) ratio, allows a good estimate of IDO activity. The local depletion of TRP and the production of proapoptotic TRP metabolites of the kynurenine pathway such as 3-hydroxyanthranilic acid and quinolinic acid are among the mechanisms potentially responsible for the immunosuppressive effects related to IDO. Since the TRP metabolic pathway is activated by pro-inflammatory stimuli, the anti-inflammatory effect of KYN metabolites provides a feedback mechanism in modulating the immune response. In contrast to other immunomodulatory KYN derivatives, such as 3-hydroxykinurenine and quinolinic acid, exerting toxicity on neural elements, KYNA is generally considered as an endogenous neuroprotective agent.

We aimed to investigate the prevalence of B7 costimulatory molecules on monocytes and that of their receptors on T cells in umbilical cord blood, adult peripheral blood and peripheral blood samples of healthy pregnant and non-pregnant women. We also determined the intracellular expression of indoleamine-2,3-dioxygenase (IDO) and the plasma levels of tryptophane (TRP), kynurenine (KYN) and kynurenic acid (KYNA), that are important molecules with immunoregulatory properties, in order to describe their potential contribution to the pregnancy-specific maternal immune tolerance and to the neonatal T cell activation.

Our findings demonstrate the previously observed lower reactivity of umbilical cord blood T cells in comparison to the adult peripheral blood T lymphocytes. Alterations in the expression of B7 costimulatory molecules as well as differences in the tryptophan catabolic enzyme, IDO activity described by our study are important regulatory factors that contributes to lower reactivity of umbilical cord blood T lymphocytes. Our investigations also suggests that the

alterations in the expression of B7 costimulatory molecules and the differences in the intracellular IDO expression and enzymatic IDO activity contribute to the development of the pregnancy-specific immune tolerance.

Therefore, the deeper understanding of the mechanisms contributing to a decreased T cell response in umbilical cord blood and adult blood immunity is of importance in improving therapeutic efficiency in related disorders.

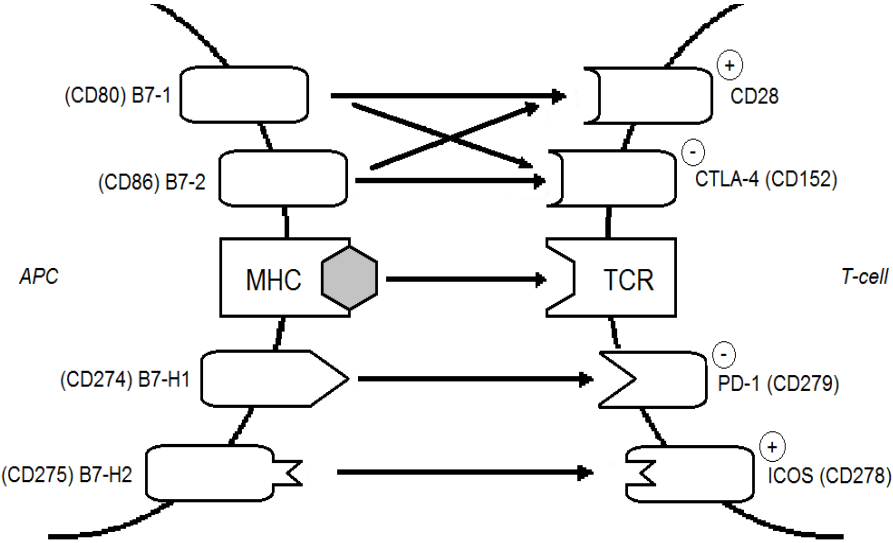

Figure 1. B7 family proteins on antigen-presenting cells (APCs) and their cognate receptors on T cells, described in details in the text. *Abbreviations: APC, antigen-presenting cell; MHC, major histocompatibility complex; TCR, T cell receptor.*

Aims

The general objective of my PhD thesis is to determine the frequency of activated (CD11b+) monocytes expressing B7-1, B7-2, B7-H1, and B7-H2, and that of T cells and CD4+ T helper cells expressing CD28, CTLA-4, PD-1, and ICOS in umbilical cord blood (UCB), adult peripheral blood (APB) and peripheral blood samples of healthy pregnant and non-pregnant (NP) women using flow cytometry (Figure 1). We also examined the intracellular expression of IDO applying flow cytometry and plasma levels of TRP, KYN and KYNA using high-performance liquid chromatography.

Methods

1. Sample collection

Cord blood samples from term neonates and peripheral blood samples from healthy adults

Peripheral blood samples were taken from 20 healthy adults and cord blood samples from the umbilical vein of 17 healthy, term neonates. Informed consent was obtained from all subjects or, in the case of neonates, parents of subjects, and our study was reviewed and approved by an independent ethical committee of the institution. The study was adhered to the tenets of the most recent revision of the Declaration of Helsinki.

Peripheral blood samples from healthy pregnant and healthy non-pregnant women

Peripheral blood samples were taken from 20 healthy pregnant (HP) women in the third trimester and 14 age-matched, healthy non-pregnant (NP) women. The latter group was synchronized in terms of menstrual cycle for the luteal phase. Informed consent was obtained from all subjects, and our study was reviewed and approved by an independent ethical committee of the institution. The study was adhered to the tenets of the most recent revision of the Declaration of Helsinki.

2. PBMC isolation

Peripheral blood mononuclear cells (PBMCs) were separated by a standard density gradient centrifugation (Ficoll Paque, Amersham Biosciences AB, Uppsala, Sweden, 25 minutes, 400 *g*, 22 °C) from freshly drawn blood collected in lithium heparin-treated tubes (BD Vacutainer, BD Biosciences, San Jose, CA, USA). Cells were kept at -80 ºC in Fetal Bovine Serum containing 10% DMSO until analysis. After thawing, cells were washed twice in phosphate-buffered saline and their viability was assessed by trypan blue exclusion (consistently > 90%).

3. Flow cytometry

PBMCs were stained for 30 min at room temperature in the dark with PerCP-conjugated CD3, PE Cy7-conjugated CD4, PE-conjugated CD28, APC-conjugated CD152 (CTLA-4), FITC-conjugated CD278 (ICOS) and APC-Cy7-conjugated CD279 (PD-1) mAbs, or PerCP-conjugated CD3, PE Cy7-conjugated CD11b, APC-conjugated CD80 (B7-1) and PE-conjugated CD275 (B7-H2) mAbs, or PerCP-conjugated CD3, PE Cy7-conjugated CD11b, APC-conjugated CD86 (B7-2) and PE-conjugated CD274 (B7-H1) mAbs in separate tubes, respectively (BioLegend, San Diego, CA, USA). After washing, cells were fixed with Fixation/Permeabilization solution and treated with Permeabilization Buffer according to the manufacturer's instructions (eBioscience, San Diego, CA, USA). They were then stained with a mouse anti-human IDO monoclonal antibody (Millipore, USA) for 30 min at 4 ºC in the dark. After washing, cells were stained with FITC-labelled goat anti-mouse antibody for 15 min at 4 ºC in the dark. After washing, cells were analyzed on a BD FACSAria flow cytometer (BD Biosciences) equipped with 488 nm and 633 nm excitation lasers. Data were processed using the FACSDiVa software. 100000 cells were recorded. The populations of lymphocytes and monocytes were gated from PBMCs according to Forward Scatter Characteristics and Side Scatter Characteristics. As control of FITC-labelled goat anti-mouse specificity staining, PBMCs were incubated with surface antibodies and FITC-labelled

goat anti-mouse antibody in the absence of mouse anti-human IDO monoclonal antibody.

4. High-performance liquid chromatography (HPLC)

The investigated reference compounds (L-TRP, L-KYN sulfate salt, KYNA) and zinc acetate dihydrate were purchased from Sigma-Aldrich (Saint Louis, MO, USA), acetonitrile and perchloric acid (PCA) were purchased from Scharlau (Barcelona, Spain) and acetic acid was purchased from VWR International (Radnar, PA, USA). Plasma samples were stored at -80 °C until analysis. Before analysis, the samples were thawed and after a brief vortex 300 µl of plasma sample was 'shot' onto 700 µl precipitation solvent (containing 3.57 w/w% PCA and 2.857 µM 3-nitro-L-tyrosine as internal standard). Following that the samples were centrifuged at 13000 *g* for 10 min at 4 °C, and the supernatant was collected.

The KYN, KYNA and TRP concentrations of the samples were quantified based on the slightly modified method of Herve et al. [16], with an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA). The system was equipped with a fluorescent and a UV detector; the former was applied for the determination of KYNA and TRP, and the latter for the determination of KYN and the internal standard. Chromatographic separations were performed on an Onyx Monolithic C18 column, 100 mm x 4.6 mm I.D. (Phenomenex Inc., Torrance, CA, USA) after passage through a Hypersil ODS pre-column, 20 x 2.1 mm I.D., 5 µm particle size (Agilent Technologies, Santa Clara, CA, USA) with a mobile phase composition of 0.2 M zinc acetate/ACN = 95/5 (v/v%) with a pH adjusted to 6.2 with glacial acetic acid, applying isocratic elution. The flow rate and the injection volume were 1.5 ml/min and 20 µl, respectively. The fluorescent detector was set at excitation and emission wavelengths of 344 nm and 398 nm, and after 3.5 min of each run the wavelengths were changed to 254 nm and 398 nm. The UV detector was set at a wavelength of 365 nm.

5. Statistics

Data are expressed as median and interquartile range. Comparisons between sample populations were made with Mann-Whitney test. Correlation analyses were performed using Spearman tests. P-values less than 0.05 were considered significant. Statistics were calculated using the STATISTICA software (version 8.0; StatSoft, Inc., Tulsa, Oklahoma, USA).

Results

Results of term neonates and healthy adults study

Higher level of CTLA-4 expression on CD4+ cells in UCB, indicating that the possibility of CD28-mediated costimulation may be decreased. At the same time, the level of the corresponding costimulator molecule, B7-2 is also elevated.

A statistically significant, but biologically modest decrease in the frequency of CD4+ CD28+ lymphocytes was observed in UCB in comparison with APB. At the same time, a more considerable increase was noticed in the expression of the regulatory receptor CD152 (CTLA-4) on these cells. The frequency of CD11b+ CD86+ monocytes was higher in UCB than in APB. The prevalence of CD3+ CD278+ lymphocytes was also higher in UCB than in APB, however, this difference was not significant in the CD4+ subset. The frequency of the corresponding costimulatory molecule, CD275 on CD11b+ monocytes was comparable in the two groups. In contrast, that of CD11b+ CD274+ monocytes, providing inhibitory signal via CD279, was lower in UCB compared to APB.

Lower capacity of UCB CD11b+ cells to produce IDO and the reverse signalling in CD11b+ cells is not mature in UCB.

A tendency was observed for an increased prevalence of IDO-expressing cells among CD3+ lymphocytes in UCB compared to ABP ($p = 0.069$), while their frequency in the CD11b+ subset was comparable. The mean fluorescence intensity (MFI) values for IDO were lower in UCB than in APB both in case of CD3+ lymphocytes

and CD11b+ monocytes. In order to explore the presence of reverse signalling via CD80 and CD86, correlation analyses were performed. A negative correlation between the frequency of CD11b+ CD86+ monocytes and IDO-expressing CD11b+ monocytes was found in UCB ($r = -0.59$), while no correlation was present in APB. Furthermore, a positive correlation was detected between MFI of IDO in CD3+ cells and the prevalence of CD11b+ CD80+ monocytes in APB ($r = 0.48$).

Plasma KYN and TRP levels were higher in UCB than in APB.
We observed a more pronounced increase in KYN, resulting in a more than two-fold higher K/T ratio in UCB compared to APB. KYNA levels were also considerably, almost ten-fold higher in UCB.

Results of HP and NP study

The expression of CD28 was increased, while that of CTLA-4 was decreased on T lymphocytes isolated from HP compared to NP women.
A significant increase in the prevalence of CD28+ T cells was observed in HP compared to NP women. At the same time a decrease was shown in the expression of CTLA-4 on these cells.

The expression of B7-1 was decreased on HP monocytes.
The prevalence of both CD278+ and CD279+ T cells was higher in HP than in NP women. The frequency of both CD80+ and CD275+ monocytes was lower in HP women, however, no difference was observed regarding CD86+ and CD274+ monocytes.

The prevalence of IDO-producing T cells and monocytes as well as the intracellular amount of IDO was elevated in HP.
The prevalence of IDO-expressing T cells and monocytes was higher in HP compared to NP women. At the same time, the mean fluorescence intensity (MFI) values for IDO were also significantly higher in both cell subsets in HP. Plasma KYN, KYNA and TRP

levels were lower, while at the same time, the K/T ratio was higher in HP than in NP women. In order to explore whether reverse signalling via CD80 and CD86 is present in monocytes, correlation analyses were performed. However, we could not detect a correlation between the frequency of CD80+ or CD86+ monocytes and the frequency of IDO-expressing T cells or monocytes or the MFI of IDO in the investigated study groups.

Conclusions

1. The level of CTLA-4 expression on CD4 cells was higher in UCB indicating that the possibility of CD28-mediated costimulation may be decreased. The level of the corresponding costimulator molecule, B7-2 was also elevated. Therefore, this inhibitory relation function is present to a higher extent in UCB.

2. The plasma KYN / TRP ratio was higher in UCB compared to APB due to the overexpression of IDO in competent cells.

3. The increased IDO activity in plasma, which is responsible for the development of the foetal maternal immune tolerance, plays a role in decreased T cells reactivity in UCB. However, the capacity of UCB monocytes compared to APB monocytes was lower to produce IDO, and reverse signalling via B7-2 in UCB monocytes was found to be immature, which suggests that the observed increase in KYN / TRP ratio may be due to placental rather than foetal overexpression of IDO in competent cells. These factors may all contribute to the previously observed reduced reactivity of UCB T lymphocytes compared to APB T cells.

4. The expression of CD28 was increased, while that of CTLA-4 was decreased on T lymphocytes isolated from HP compared to NP women. The expression of B7-1 was decreased on HP monocytes.

5. The development of the pregnancy-specific immune tolerance in the mechanism of B7 costimulation may be more related to the altered expression of B7 proteins on APCs rather than that of their receptors on T cells.

6. The elevated intracellular IDO expression in monocytes and T cells, as well as the higher plasma enzymatic IDO activity probably contributes to the systemic immunosuppressive environment in the third trimester characteristic for healthy gestation.

Bibliography of the candidate's publications

Publications related to the thesis:

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