Review paper

Distinctions of the neonatal immune system

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Abstract

On the grounds of a wide scope of cited actual literature, the article describes specificity of the immune system in peri-natal period of life. After presentation of the most basic tasks of the system at the time of transition from intrauterine into the extrauterine environment, the authors describe in multiple details how the new-born's immune system attains the ability to cope with the new tasks. The main differences in the mechanisms of immune reactivity between the neonatal and adult immune systems are demonstrated. In particular, the development of innate and acquired immune functions are outlined including the role of neutrophils, adhesins, complement cascade, cytokins, numerous antigen presenting cells, different subclasses of T lymphocytes (Th1, Th2, Tregs) and B cells at their stages of naive and engaged cells. The role of passive transfer of humoral immunity from mother is also described. The authors suggest that in contrast to the common opinion on functional disability of neonatal immune system, the system represents the specificities characteristic to the gradual transition from the general tolerant to the active defensive state with preservation of self-tolerance.

Key words: *neonatal immunity, adult immunity, differences.*

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Upon delivery, each organ and system of the newborn's body is a subject of exposure to the extra-uterine environment, dramatically different from the previous safe and comfortable conditions. This transition encompasses the whole series of events, described as maturation in progression of age. There are three major tasks faced by the fetal, and then neonatal immune system: prevention of infections, including those which develop at junction of maternal and fetal tissues; avoidance of the destructive influence of Th1 lymphocytes – the cells with pro-inflamatory functions capable of inducing allogenic immune reactions between mother and fetus ; finally, maintaining balance between the sterile intra-uterine environment and the abundance of third-party antigens present in the outer world, which requires the primary colonization of skin and alimentary tract by microorganisms [1-4].

According to the WHO statistics, approximately 7.1 million neonates and infants die each year due to infections, including over 2 million during the first 6 months of life. The main causes of death are infections of viral and bacterial origin affecting the respiratory and alimentary systems [5, 6]. This is aggravated by the immaturity of the immune system, reflected by the not-fully developed structures of bone marrow, spleen and lymph nodes, low number of B and T lymphocytes and of B and T memory cells. The state of immunodeficiency is completed by the quantitative and qualitative defect of antigen-presenting cells [7, 8]. The varying level of immaturity concerns all the components of the immune system, responsible for evolution of innate and acquired defence mechanisms.

The first line of defence against pathogens is composed of neutrophils, monocytes, macrophages, dendritic cells and complement components. The majority of leukocytes in the newborn's blood are neutrophils (70- 90%), expressing the surface receptors FcαRI, FcγRIIA and FcγRIIC, which allows them to bind the maternal antibodies and consecutively antigen-antibody complexes [9]. In full-term newborns, the number of neutrophils at 12 hours of life varies between 7 and 15 G/l, and is higher in those born prematurely. During the first 24 hours after delivery the number of neutrophils in peripheral blood grows rapidly, stabilizing between 48 and 72 hours of life. This transient increase

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in number of neutrophils is reflected by the elevated value of neutrophil progenitor cells and their gradual decline during the first week of life. The absolute neutrophil number, comprising their mature and progenitor forms, is higher in newborns compared to adults [10-16].

The neutrophil progenitor cells are characterized by limited proliferatiive ability towards mature neutrophils in response to antigen stimulation, especially in case of premature infants. Neutropenia, which accompanies the newborn's bacterial sepsis, is an indicator of unfavourable prognosis [17-20]. The quantitative deficiencies and decreased proliferative ability in response to antigen stimulation are often accompanied by impairment of such cellular functions as adhesion ability, engulfment, chemotaxis, phagocytosis and intracellular killing [20, 21].

The reason of defect in adhesion ability of neonatal neutrophils seems to be the low expression of CD18/CD11b complex, a member of β2 integrin family. The low level of CD11b molecule expression correlates with the defect of neutrophil adhesion to endothelium. This in turn is a reason of recurrent bacterial infections [1, 7, 22].

The other factor determining the leucocyte adhesion is L-selectin. In newborns, the L-selectin expression is lower as compared to those in adults. Its expression decreases during the first 24-72 hours after delivery in parallel to transient leukocytosis, and in full-term newborns is lower in comparison to adults and premature infants. The lowered expression of L-selectin correlates with the impaired ability of neutrophils to "roll" over the endothelial surface, which is crucial in initiation of adhesion process [23-25].

Along with the lower adhesion ability, neutrophils respond weakly to antigen stimulation, due to the impaired calcium metabolism and incorrect polymerization of actin. As measured in cord blood, the concentration of guanosinetriphosphate binding protein (Rac2), the main regulator of migration and chemotaxis of human neutrophils, is significantly lower than in adults. The decrease of neutrophil migration ability is suggested to present a risk factor in development of severe bacterial infections in premature newborns, despite the preventive antibiotic treatment of their mothers [24, 26].

The unfavourable condition observed in neonatal period, especially in premature neaonates, with accompanying disturbances in phagocytosis, is a decreased opsonin activity, including fibronectin, immunoglobulins and lectin components of complement activation [27, 28].

There are over 40 proteins which form the complement cascade, activating the classical, alternative and lectin pathways. The complement components take part in phagocytosos, facilitate lysis of bacteria and activate naive B lymphocytes. The complement cascade activation results in synthesis of inflammation mediators (C3a, C5a), opsonization of cells (C3b, C4b), and macrophage-mediated phagocytosis through formation of membrane attack complex (C5b-9) on target cells [29]. In consequence, the modulation of activity of neutrophils and complement components may lead to excessive pro-inflammatory response, causing damages in engaged tissues.

The complement proteins concentration rises after delivery, reaching values comparable to that in adults around 6-18 months of life. The reference values for C1r, C2, C5, C7, properdin, factors D, H, I, C3a and C5a are evaluated in cord blood on full-term infants. In premature ones, even in absence of infection symptoms, the concentration of complement proteins is lower than in mother's serum.The classical route of complement activation (C3) is fully complete already in week 25 of fetal life. The C3d component is the most sensitive marker of inflammation of placenta [30, 31].

During the neonatal and infantile periods, the functional defect of mononuclear phagocyting cells is observed. It concerns mostly the low synthesis of cytokines and lowered phagocyting capacity. The newborn's monocytes reveal low expression of co-stimulating molecules – CD86 and CD40, which remains stable even after stimulation with IFN-γ or the CD40 molecule ligand (CD40L) [32]. In addition, the newborn's monocytes and macrophages synthetize little amounts of pro-inflammatory cytokines: TNF, IL-1 or IL-2 in response to stimulation with bacterial antigens like LPS (TLR4 ligand), mycoplasma-associated lipopeptide (TLR2/6 ligand) and immiquimod (TLR7 ligand) [33-35]. These deficiencies result probably from defective pathway of transducing activation signals via TLR receptors, though the exact nature of such defects has not been defined yet. The expression of mRNA, encoding various TLR receptors in newborn's monocytes, reaches levels comparable to adult persons which do not necessarily explain the differences in expression of proteins forming TLR receptors. Hence, following the stimulation of newborn's monocytes with LPS, no increase in expression of TLR4 receptor and CD14 molecule has been observed, and the level of expression of MyD88 – the adaptor molecule involved in signal transduction from TLR receptor – ultimately drops. The concentration of mRNA encoding other molecules responsible for transduction of TLR receptor signal, namely TIRAP and IRAK-4, is also comparable to this in adults, but their protein product levels in newborn's monocytes remains undetectable. The gradual evolution of mechanisms responsible for antigen binding by TLR receptors is further supported by the fact, that expression of TLR4 receptor proteins in premature newborns is lower than in full-time ones. This is one of explanations of the increased susceptibility of immature neonates to infectious agents, and a proof, that the regulation of TLR receptor expression originates in the fetal life [36-41].

One of the monocytes' functions is phagocytosis. Its immaturity in the neonatal period is reflected, among others, by diminished phagocyting ability towards *Escherichia coli*, as compared to adult individuals. The gradual maturing of the phagocyting functions is supported by the fact, that newborns delivered before the 30th week of pregnan-

cy show only trace levels of phagocytic activity. This phenomenon does not seem to affect pinocytosis, where it has been proven that for instance the level of bovine serum albumin uptake is close to that observed in adults [29, 42].

The neonatal macrophages show also low sensitivity to stimulation with IFN-γ, which affects the ability of intracellular killing of pathogens. In monocytes isolated from cord blood, the low synthesis of IL-2 in response to stimulation with *Staphylococcus aureus Cowan* (SAC) antigens has been observed [19, 43, 44].

The key role in initiation of specific immune response is performed by antigen-presenting cells (APC). The final stage of immune response is mostly dependent on the APC type. Naive T cells require dual signal for their activation. One is received by TCR receptors, while the other is a co-stimulatory signal transmitted *via* B7 receptor family present on APC plus CD28 and CTLA-4 receptors expressed by lymphocytes. These signals are amplified by the action of humoral factors – cytokins, which present the third stimulus in the process of activation of naive T cell. Therefore, the interaction between APC and T lymphocytes is a mutual dialogue, in which the antigen presenting cell must possess appropriate mechanisms to stimulate the T cell, and the latter one must be equipped with adequate receptors enabling the reception of activating signal [45, 46].

The immaturity of the neonatal immune system involves also antigen presenting cells, including dendritic cells. Due to their scarce presence, most of the studies is carried out *in vitro* on so called monocyte-derived dendritic cells (MDDCs). In cord blood, dendritic cells (CBMDDCs) form around 0.3% of tne mononuclear population. They are characterized by low expression of co-stimulating molecules (CD80, CD86, CD40) and MHC class II antigens, delayed maturation of both myeloid and plasmacytoid progenitors, limited ability to respond to alloantigens and defective endocytosis. In these cells the lower synthesis of IL-12 is observed, and the levels of such cytokines as IL-6, IL-8 or TNF are comparable to those in adults, while IL-23 is even higher. The cord blood dendritic cells are further specified by low expression of CD1a molecule – the receptor active in presenting the lipopeptide antigens. Following LPS stimulation, CBMDDCs reveal the phenotype of immature cell, with low expression of HLA-DR, CD86 and CD83 [47-49].

The reasons for neonatal dendritic cell immaturity are associated with disruptive conveying of activation signal from TLR receptors. It has been shown, that after LPS stimulation the levels of mRNA for proteins responsible for transmission of activation signal, like MAPKKK, NF-κB and TANK, are significantly lower in neonatal dendritic cells as compared to corresponding values in adult persons [26, 49].

The next feature revealed by the neonatal dendritic cells is a functional immaturity of endoctosis, most probably caused by the low expression of mannose receptor and low synthesis of IL-12 in response to stimulation with LPS and CD40L. There is an assumption, that one of the reasons for functional deficiency of neonatal T lymphocytes may come from the weakness of co-stimulatory signal. The experiment, in which allogenic cells evoked significantly lower response of neonatal MDDCs vs their adult counterparts, as measured by proliferation and IFN-γ synthesis, has been cited to support this hypothesis [50, 51].

Due to their functional immaturity, neonatal APC require for their activation a stronger stimulating signal than these cells in adults. Experimental studies reveal the involvement of signal transmission cascade from TLR receptors in different subopulations of APC in newborns. Upon adequate activation, APC cooperate with CD4+ lymphocytes, but the insufficient activation of T lymphocytes brings reversily the defective APC functions. One of the reasons of functional defects of dendritic cells is believed to be an excessive activation of regulatory T cells. The inhibitory effect of thymus-derived nTreg lymphocytes on dendritic cell activity has been demonstrated in murine experimental system. The suppressive effect of nTregs on APC can be also mediated *via* inhibition of effector T lymphocytes and/or synthesis of suppressive cytokines, like IL-10 and TGF-β [25, 52, 53].

In the fetal life the hematopoiesis takes place in liver, pre-B lymphocytes with cytoplasmic expression of IgM are detected in the 8th week of pregnancy, and in week 10-12 pre-B lymphocytes with surface expression of IgM are observed. Over 90% of B lymphocytes from fetal spleen shows CD5 receptor expression, which is also a dominant population in cord blood. T lymphocytes isolated from cord blood differ in phenotype from the naive lymphocytes evaluated in peripheral blood of adult individuals. They are mostly represented by cells in early stages of development with high level of TREC expression. Dendritic cells with MHC class II expression are found in fetal liver and thymus [3]. In the 12th week of fetal life their presence is also detected in lymph nodes. The colonizing dendritic cells appear in skin and throat lymphoid tissue around week 23 of pregnancy. Like in adult life, B cells require stimulation from T lymphocytes for proliferation and antibody production to take place [19, 46].

The immaturity of the cellular component of the newborn's immune system springs out mostly from the low proliferation ability of T lymphocytes, lowered synthesis of IL-2, diminished cytotoxic activity, insufficiency in boosting B cell response and altered profile of synthesized cytokines, as compared with adult age [41, 54-56].

The maturation of immune system is a dynamic process, which is influenced by various external factors, just to mention the humoral agents of the maternal milk [57]. While investigating the potential protective means of immunity, we must not forget that every pathogen triggers separate, individually specific elements of this system. For obvious reasons, the majority of observations on antigen action exerted on the immune system were based on murine exper-

imental model. It has been shown, that T cell response to intact or attenuated viral antigens differs between neonatal and adult immune systems. In consecutive studies, the attempt was made to evaluate the favourable conditions for the development of infectious diseases. The inoculation of Cas-Br-E virus from the murine leukemic viral family (Cas) caused infection in experimental animals, with resulting neuro-degenerative syndrome leading to tetraplegia within 6-8 weeks. In the parallel study, conducted on adult animals, no such effect has been observed. In contrast, the increased activity of cytotoxic cells with CD8 phenotype, as measured by elevated synthesis of IFN-γ has been noted. No such correlation was observed in murine neonates. However, the supplementation of these neonates with „adult" CD8 cells resulted in restoration of immunity against the inoculated virus, with no subsequent clinical manifestation of the disease [57-59].

The principal task of T cell subset is to achieve the ability to recognise "self" and "non-self". This ability is acquired in thymus, where the elaboration of complete repertoire of T cells takes place, as a result of T-cell receptor rearrangement in context of self MHC class I and II antigen restriction, and following the dual (positive and negative) selection process. This way, T lymphocytes build gradually their competence in perception of "self" structures and, consequently – altered "self", which forms the basis for the eventual neutralization of negative influence of external factors.

There is a bulk of evidence showing, that exposition to a patogen *in utero* leads to recruitment of pathogen-specific fetal T lymphocytes. The increase in number of antigen-specific T lymphocytes was evaluated in children with innate or neonatal infection with cytomegalovirus (CMV) or herpes virus (HSV), however – reversely to adults – HSV infection in neonates results in recruitment of specific CD4+ lymphocytes synthesizing IFN-γ. During CMV infection the number of specific CD4+ lymphocytes in neonates is significantly lower than this in adults, but the number of CD8+ lymphocytes is comparable [28, 44, 52, 57]. These observations suggest, that antigen stimulation of sufficient intensity may induce the T cell response even in the neonatal period.

Peripheral T lymphocytes evaluated in newborns differ significantly from those in adults, and possess the phenotype of early cells derived from the thymus. They are characterized by the presence of TREC – side effect of TCR receptor rearrangement process, indicator of the T cell proliferative activity [3]. Like in adults, most of the neonatal naive T lymphocytes present the phenotype of CD45RA+ cells, with co-expression of co-stimulatory molecules CD27 and CD28. In contrast to the adult population, there is a high expression of CD38 receptor on the surface of naive neonatal T lymphocytes. The vast majority of neonatal T lymphocytes undergoes proliferation, becoming more susceptible to apoptosis. The multiplication of naive T cells takes place in fetal life and reaches the plateau at the age of around 5 years. This high degree of proliferation observed in the neonatal period plays probably a key role in defining the repertoire of T lymphocytes. Apart from the high proliferation rate, there is a high level of telomerase observed in the neonatal T lymphocytes, which prevents shortening of telomers during the subsequent cell divisions.

During the *in vitro* experiments, the apoptosis of T lymphocytes could be blocked by means of cytokine stimulation of the common chain of the IL-2, IL-7 and IL-15 receptors. The two last cytokines induce the proliferation of the neonatal T lymphocytes, without presence of any other stimulatory signals. Interleukin 7 is a cytokine required for intra-thymic T lymphocyte maturation [19, 24, 46, 52, 60]. The neonatal circulating T lymphocytes are characterized by high expression of α chain of IL-7 receptor – CD127 molecule, which is not observed in adult persons. Interestingly, the induction of proliferation of neonatal T cells is dependent on the activity of caspases, which mediate the apoptosis process. Interleukin 7 does not stimulate the intrathymic proliferation of T lymphocytes. This role is performed by IL-15, which stimulates the proliferation of CD8+, but not CD4+ lymphocytes, what has been unequivocally proven in *in vitro* studies. In the process of maturation and differentiation of T lymphocytes, the key role is attributed – along with IL-7 – to the degree of interaction between MHC class II complex and TCR receptor [3, 19, 47].

Th1 lymphocytes synthesize the cytokines responsible for inflammatory reaction. Because of the risk of placental damage, there are mechanisms during pregnancy to restrict the activity of Th1 cells. One of such factors is IL-10, synthesized by fibroblast, the other – progesterone [31]. In neonates, the Th1-mediated type of response remains significantly lower than that evaluated in adult life.

The neonatal CD4+ cells produce little amounts of IFN-γ which is hypermethylated in CpG and non-CpG sites in IFN-γ precursor [39]. Upon sub-optimal stimulation *via* CD28 receptor, the newborn's CD4+ lymphocytes are induced to produce both IL-4 and IFN-γ, while in adults it concerns only IFN-γ [46].

In response to polyclonal or super-antigen mediated stimulation, the neonatal CD4+ T lymphocytes synthesize cytokines of Th2 profile, whereas IL-12 induces production of IFN-γ. In turn, neonatal CD8+ lymphocytes synthesize IFN-γ in quantities comparable to those in naive lymphocytes of adult persons.

The functional differences between T lymphocytes in newborns and adults seem to result mosty from the way of transmission of activating signal. Upon activation, the former ones reveal significantly lower expression of transcription factors, like nuclear factors of activated T cell (NFAT), CD154 genes or IFN-γ [19, 46]. The most significant difference between these two groups, however, seems to be the mode of their transformation from dually-positive (CD4+CD8+) lymphocytes into phenotypically defined

CD4+ thymocytes. In cord blood, these cells possess the phenotype of early thymic emigrants with high expression of chemokine-binding receptor (thymus-expressed chemokine (TECK), like CCR9 [30, 39].

The regulatory mechanisms of T cell reponse are elaborated during the first years of life. One component constituting this system is a regulatory T lymphocyte subpopulation (Tregs), with CD4 cell phenotype and a co-expression of IL-2 receptor – CD25 molecule. Differently than in adults, Tregs in newborns are characterized by naive CD45RA cells phenotype, Tregs lymphocytes inhibit the proliferation of effector CD4 and CD8 lymphocytes, thus preventing the onset of autoimmune and allergic processes, but also hampering the anti-tumour response. T regs are represented in high proportion in premature newborns, which suggests their role in development of the fetal immune system. The regulatory features are also acquired by CD4+ cells cultured *in vitro* in a medium supplemented with IL-10 and IFN-α [19, 46, 61].

A useful model for evaluating the impact of antigens on the developing immune system is investigation of changes brought about by vaccination. Newborns immunized perinataly by vaccination against viral hepatitis B (HBVac) and *per os* by a vaccine against poliomyelitis (OPV) develop Th1 type response much weaker than this in adult persons [22, 34, 36]. The magnitude of this response correlates conversely with much higher production of antibodies than in adults. In the early stage of the response, the synthesis of Th2 type cytokines is comparable, while the synthesis of cytokines by the memory cells in response to HBVac is higher in newborns than in adult persons. The weaker neonatal Th1 type response to vaccinal HBVac and OPV antigens stays in contrast with mature Th1 type response induced by BCG antigens administered in the same period. Such response, evoked in the perinatal period, is comparable in its magnitude and quality to the one achieved in adults after BCG vaccination. *Mycobacteria* and *Bordetella pertusis* stimulate the dendritic cells (DC) which in turn recruit the naive T lymphocytes to the immune response [22, 29, 34, 36].

Due to the immaturity of immune system, the active immunization of neonates appears relatively inefficient, which is reflected by low synthesis of antibodies against the vaccinal antigens. With exception of BCG, most of the vaccines are administered in divided doses, proportionally to the age and level of maturity of the immune system [34, 35].

The course of neonatal infections is mostly reliable on antibodies delivered in fetal life by the mother, which creates the opportunities for passive immunization of neonates [44].

For the first time, the protective role of maternal antibodies was observed in 1846 in Faroe Islands, during the outbreak of measles. The children of mothers, who went through the disease, did not fall sick during the neonatal period. The next observation concerns the vaccination of mothers against smallpox, also effective in protection of their children [26, 52].

The transportation of IgG from the mother's circulation is an active and selective process, mediated by FcRn receptor and restricted only to IgG – IgG1 and IgG3 subclasses. The transfer of maternal IgG starts in the 17th week of pregnancy, in week 33 the concentration of IgG in fetal serum is comparable to the mother's one, and in week 40 even prevails. Maternal IgG provides protection throughout the first several months of the newborn's life.

The other humoral factor, delivered to the newborn in a passive way and present in mothers milk, is a secretional form of IgA. SIgA is present in mother's milk in concentration between 0.5 to 1.5 g/l. The milk contains also limited amounts of IgG and IgM. SIgA is mostly active in protection of intestinal mucosal membrane against such pathogens as *Shigella*, *Vibrio cholerae*, *Campylobacter*, *Enterotoxigenic Escherichia Coli* (ETEC) or *Giardia liamblia* [41, 55, 62, 63, 64].

Another humoral agent delivered by the mother's milk is lactoferrin (LF), present in concentration of around 1-4 g/l. Likewise SIgA, it is relatively resistant to enzymatic degradation. In human milk, SIgA and LF constitute together approximately 30% of all the proteins, whereas in the bovine milk only 5%. In addition to its protective role against pathogens, LF reveals also the immunostimulating and anti-inflammatory functions, through its action on the synthesis of IL-1β, IL-6, TNF and IL-8 [25, 27].

The fetal, and subsequently neonatal immune system must deal with many challenges, like prevention of infections from one side, and avoiding excessive pro-inflammatory responses from the other. It must also cope with the transfer from the sterile intra-uterine environment to the pathogen-abundant external one. In the first stage, the efficient functioning of the immune system relies mostly on the non-specific elements composing the so called innate immunity – which gives time for elaboration of the sophisticated mechanisms of specific immune responses. The quantitative and functional potential of the developing neonatal immune system makes it possible, under some circumstances, to generate the response equal to that observed in adult persons [27, 30, 57, 65].

This seems to question the thesis about severe deficiencies of the neonatal immune system [3, 26]. In contrast to the common opinion on functional disability, the system represents specificities characteristic to the gradual transition from the general tolerant to the active defensive state with preservation of self-tolerance.

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