Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

SCIENZE MEDICHE GENERALI E DEI SERVIZI

Ciclo XXVII

Settore Concorsuale di afferenza: 06/G1

Settore Scientifico disciplinare: MED/38

EVALUATION OF IMMUNE FUNCTION IN PRETERM INFANTS USING IMMUKNOW® ASSAY

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Esame finale anno 2016

SUMMARY

1. INTRODUCTION

Preterm infants are exposed to a high risk for infection by a wide range of viruses, bacteria, protozoa, and fungi in the neonatal period. Infectious disease is one of the major causes of mortality and morbidity among newborns and it accounts alone for more than 50% of the deaths in infants born with gestational age <28 weeks.

The need of intensive care with frequent invasive procedures and the immaturity and inexperience of the neonatal immune system are the main predisposing factors.

However the characteristics of the preterm infants immune system are not fully understood as well as the factors that can influence immunity in the perinatal period.

It has been demonstrated that preterm infants have an adequate, even higher, number of leukocytes and lymphocytes compared to adults; however these numbers do not reflect the level of immunological competence of the subjects since these cells may lack of function at birth.

Lymphocytes, especially CD4+ T cells, play a crucial role in the regulation of the immune system. Because they are involved in the modulation of both humoral and cell-mediated adaptive immunity and they also have a relevant part in the control of innate immune response, they are often targeted as a marker of the global immune function.

The aim of the study was to assess the functional activity of CD4+ cells at birth and at 30 days of life and to assess the influence of the main perinatal factors in their level of activity. In order to investigate the CD4+ T cells fucntion we used the Immuknow® assay that quantifies the levels of ATP after in vitro stimulation. This test has previously only been tested in adults and children and this is the first study that uses this assay to assess T cell mediated immunity in preterm infants.

2. THE DEVELOPMENT OF THE IMMUNE SYSTEM

The ontogeny of immune system starts early in the embryo, continues during fetal life and is completed only several years after birth. [1]

At the time of birth, the immune system has not fully matured. The adaptive immune system must still develop specificity and memory, which is completed only in the early childhood years [2]. Therefore newborns rely heavily on their innate immune response which is also immature. [3]. Preterm infants have even more pronounced deficiencies in both innate and adaptive immunity, and in the interaction between these two systems. [4,5]

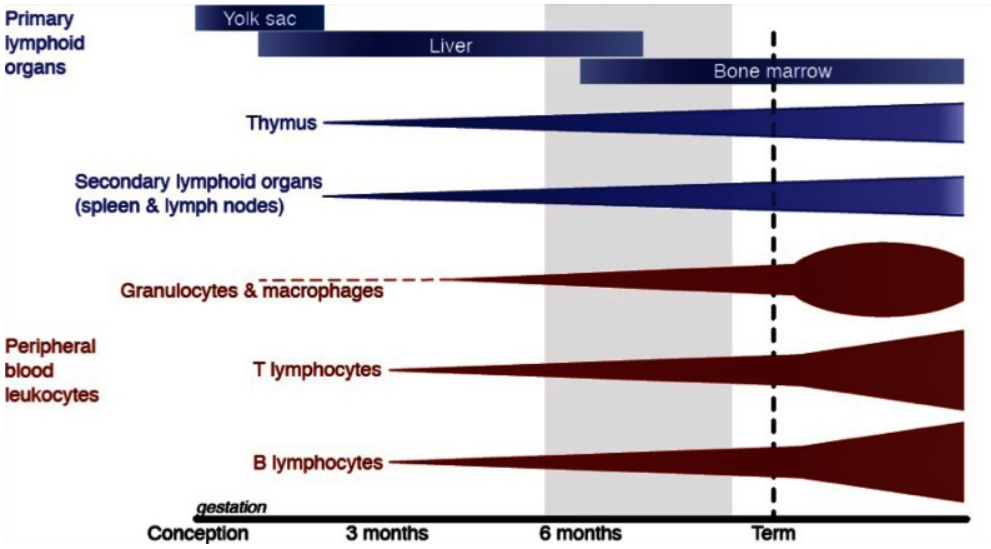


Figure 1 Leukocyte development. In grey: preterm birth. [6]

2.1Innate immunity

Neutrophils

At the time of birth infants have a lower number of neutrophils compared to adults. This deficit is more pronounced, the lower the gestational age is at birth. The pool of neutrophil progenitors is also small and can be readily exhausted during sepsis [7]

Within few hours from birth the neutrophil count peaks and then gradually decreases during the following 48-72 hours to reach the normal range of 7.000 –1000 cells/μL, in absence of complications. [8]

It appears that not just the number of neutrophils but also their functions are impaired at birth. Compared to adults, neutrophils from both term and preterm infants adhere poorly to the endothelium due to a lack of selectin expression. which limits the recruitment of circulating neutrophils into the tissues.[9] Selectin expression is further reduced by perinatal stress such as in birth asphyxia [10]. Moreover neutrophils from both term and preterm neonates display a reduced deformability and have an impaired chemotactic response, migrating at only about half the speed traveled by adult cells. [11-13]

While neutrophils from term infants achieve normal chemotactic function by 2 weeks after birth, postnatal neutrophil maturation proceeds very slowly in preterm infants [14] and in infants <34 GA is still impaired at 42 wks PCA. [15] Although minor infections may enhance chemotaxis in neonates, the migratory responses of neonatal neutrophils may become further depressed during systemic Gram-negative sepsis. [16]

Studies have shown that preterm neutrophils also have impaired phagocytosis reduced opsonic activity and generate a depressed respiratory burst which is the major killing mechanism in neutrophils. [12,17]

The neutrophil respiratory burst in infants born at 24–28 weeks is clearly less robust than in those born at 29–35 weeks and takes about 2 months to correct. However, neutrophils from preterm infants continue to have an overall weaker oxidative burst than adults and may not show any improvement in critically-ill preterm infants. [18] Neutrophils from term neonates have granule contents and degranulation responses similar to adults. [19]

However, neutrophils from preterm infants have a lower capacity to release BPI, elastase, and lactoferrin than in adults and term infants. [12,20]

Collectively, neutrophil line immaturities and limited function account for a substantial component of neonatal susceptibility to invasive bacterial infections.

Monocytes and Macrophages

Monocytic cells can be detected early during gestation. They are first seen in the yolk sac at 3–4 weeks of gestation and after few weeks they can be detected in the the fetal liver. During the second month of gestation, as hematopoiesis becomes established in the fetal liver,

monocytes are seen in high proportions and constitute nearly 70 percent of all hematopoietic cells. Over the next 6 weeks, as the erythroid cells predominate, this proportion falls to 1–2 percent. The first monocytes in circulation are not seen until about the fifth month of gestation [21], and remain uncommon until the bone marrow becomes the predominant site of hematopoiesis [22]. At 30 weeks, monocytes comprise 3–7 percent of hematopoietic cells [23]. Term cord blood studies show a relative monocytosis, which persists during the neonatal period. The absolute monocyte counts tend to decline gradually from 1340–2200/μL in the first week to about 700 in the third week [24].

Unlike neutrophils, the major host defense functions of monocytes in cord blood of term infants are largely intact. [25]. Cord blood monocytes show adherence, random migration, chemotaxis, bactericidal activity, phagocytosis-associated chemiluminescence, production of superoxide anion (O-2) and generation of hydrogen peroxide at levels comparable to adults [26, 27]. The ability of fetal and neonatal monocytes to kill a variety of pathogens including *S. aureus*, *S. epidermidis*, *E. coli*, and *C. albicans* appears to be equivalent [26, 28].

Information on the number and function of tissue macrophages in the neonatal period is little and mainly derived from autopsy studies. The size of the macrophage pool varies in different organ systems. In the gastrointestinal tract, macrophages appear in the lamina propria as early as 10 weeks of gestation and a sizable macrophage population can be seen during midgestation [29]. In contrast, the alveolar macrophage population remains small in the fetus and expands rapidly during the early neonatal period presumably as a result both from an influx of monocytes from the circulation as well as from clonal expansion in situ. [30] One can speculate that the number of monocytes, despite being adequate at birth in a term newborn, can be quite low in the preterm infants. [31]

In terms of function it appears that cytokine production is different in preterm infants: while IL-1 e TNFα concentrations are similar to adults, IL-12 ed IFN-γ production is impaired. These cytokines are necessary for the stimulation and activation of Th1 lymphocytes and to establish an adequate immune response against intracellular organisms and viral agents. [32]

2.2Adaptive Immunity

Adaptive immunity, involving lymphocytes (B and T cells), is pathogen-specific and requires acquisition of immunological memory. Maturation of adaptive immunity occurs mostly after term birth, so all newborn infants have deficiencies in T cell activation and cytokine

production, B cell immunoglobulin production, and interactions between T and B cells, relative to adults. [6]

Dendritic cells

Dendritic cells (DCs) are a discrete leukocyte population with a highly developed antigen-presenting function. Cells with a dendritic/macrophage structure are present in the yolk sac, mesenchyme and the liver at 4–6 weeks of gestational age. DCs are detectable in skin by 6–7 weeks of gestation. [25]

Cord blood-DCs represent about 0.3% of all mononuclear cells. Due to the low frequency of DCs in peripheral blood, most studies of neonatal DCs have been carried out using *in vitro* monocyte-derived dendritic cells (MDDCs). As for the macrophages it seems that their function as stimulators of lymphocytes function is impaired due to a lack of production of cytokines and a low expression of co-stimulatory molecules. [33].

T-Lymphocytes

The thymus arises at about six weeks of gestation from the third branchial arch, with the cortex arising from its ectodermal layer and the medulla from the endoderm. Lymphoid cells migrate over the next 2–3 weeks, initially from the yolk sac and fetal liver, and then from the bone marrow to colonize the fetal thymus [34]. These prothymocytes interact with the stroma, proliferate actively, and are triggered to differentiate with expression of the first T-cell-specific surface molecules (e.g., CD2, and later CD4 and CD8) [35,36]. A clear delineation of the thymic cortical and medullary regions occurs at 12 weeks of gestation; Hassall's corpuscles appear shortly thereafter [37,38]. The most immature thymocytes are found in the subcapsular cortical region, and cells move into the deeper layers as they mature [37]. The early prothymocytes do not express CD3, the T-cell receptor (TCR), CD4, or CD8 and are often referred to as triple-negative thymocytes [39]. The progeny continue to divide and rearrange their TCR genes, and since these cells express both CD4 and CD8, they are now called double-positive [37,39]. They undergo positive selection by self-major histocompatability complex (MHC) restriction, and more than 95 percent (about 50 million) cells die each day during this stage [39]. Negative selection occurs next, and is mediated by the bone marrow-derived antigen-presenting cells (APC) (e.g.,dendritic cells and macrophages), which eliminate autoreactive cells either by clonal deletion or clonal anergy

[40]. As these thymocytes mature and reach the medulla, they express only one of the CD4 or CD8 antigens. These single-positive T-cells migrate from the thymus to the peripheral lymphoid organs at about 14 weeks of gestation [37]. By 15 weeks, human thymocytes express a complete set of TCRs [37, 41]. During fetal life, thymus is the largest lymphoid tissue in terms of body proportions. It is about two thirds its mature weight at birth, and reaches its peak mass at around 10 years of age. Subsequently, it continues to involute and is replaced by adipose tissue [42].

From 19 weeks of gestation T-cell subpopulations gradually increase in number and continue to rise after birth to peak at about 6–9 months. The numbers subsequently decline, and adult levels are finally reached at 6–7 years of age [43]. In term neonates, CD4+ cells constitute a higher proportion of T-cells than adults. CD8+ cells, on the other hand, are fewer both in terms of their absolute number and as a percentage of total T-cells. Therefore the CD4/CD8 ratio is as high as 4.9:1 during the perinatal period, and declines to adult values of approximately 2:1 only by 4 years of age [43].

Preterm infants have a significantly higher number of CD4+ T-cells while the number of CD8+ T-cells does not seem to change with gestational age [44].

Around 80–90% of T-cells in cord blood are naïve lymphocytes (CD45RA) compared with only 40–60% of in the adults. The percentage of memory T-cells (CD45RO) increases in healthy infants during the first few years, but reaches adult levels only later in life [45].

Cord blood T-cells from premature infants have a limited capacity for mitogen-induced proliferation but these defects are corrected by full-term [45,46].

Neonatal concentrations of pro-inflammatory cytokines such as IL-1, IL-6, TNF-α, IFN-α, and IFN-β are comparable to adults, and also increase similarly during sepsis [47,48]. Premature infants, however, produce lower amounts of TNF-α and IFN-α compared to those born at term [49,50]. Among the cytokines involved in adaptive immunity, only IL-2 concentrations are comparable; on the opposite IL-4, IL-5, IL-10, IL-15 and IFN-γ are known to be significantly lower than adults [49-52].

CD4+ cells

During development, naive T-cells differentiate into distinct effector T-helper (Th) subsets, a process in which cytokines play a critical role. These differentiated T-cells were originally categorically designated Th1 and Th2 cells based on distinct functional properties and the cytokines that drive their development [53]. Th1 type cytokines, such as IFN-γ and IL-2 play

a key role in initiating early resistance to pathogens, and induction of cell-mediated immunity. Th2 cytokines drive the system toward immune tolerance rather than toward defense from microbial infections. Accumulating evidence suggests that Th1 responses in newborns are compromised at several steps, including deficient production of Th1 type cytokines by neonatal CD4+ T-cells and hyporesponsiveness of neonatal macrophages to stimulation by IFN-γ. These deficiencies contribute to the apparently weak cellular immunity in newborns biased towards a Th2 type response [54]. However neonatal T-cells respond well to certain antigens such as tetanus/diphtheria toxoids, influenza, and mycobacterial antigens [46]. In response to superantigens, cord blood T-cells produce lesser amounts of IL-2 and, unlike adult T-cells, are unable to respond if restimulated with the superantigen. [55] The predominant Th2 phenotype in utero followed by a Th1 switch after birth is believed to be a key process to maintain a high level of immunologic suppression in both the mother and the fetus to enable continuation of pregnancy.

Th17 cells are a distinct CD4+ population that have been shown to play a pathogenic role in allergic, autoimmune and other chronic inflammatory diseases [56,57]. The main characteristic of Th17 cells is IL-17 production, a cytokine that promotes pathogen clearance by enhancing neutrophil recruitment to sites of infection and activating macrophages [56-58].

CD8+ cells

Cytotoxic lymphocytes (CTLs) are CD8+ cells specialized in the defense against intracellular infections and also involved in allograft rejection and tumor cell surveillance [59]. CTLs utilize two well-established mechanisms for cell lysis, one involving release of extracellular mediators (such as the pore-forming perforin/granzyme system), and a second fas/fas ligand dependent pathway that leads to target cell apoptosis [60].

CTL cytotoxicity is evident by 18 weeks of gestation, but is far less efficient that adult cells even at term (<20% of adult CTL activity) [61]. Perforin expression in neonatal CTLs is about 30% of adult levels. Circulating inhibitors such as a-fetoprotein and prostaglandins may also lead to lower CTL activity in neonates [62].

γδT-Cells

The γδ T-cells are a separated functional subset that generally do not express neither CD4 nor CD8 on their surface [63]. These cells are detectable in the fetal thymus and liver at 6–8

weeks of gestation, and are nearly 10% of the peripheral blood T-cells at 16 weeks [64]. Subsequently, the numbers decline gradually to reach about 3% at term [65].
These cells are present mainly on skin and mucosal surfaces [63]. Although the exact functions of these T-cells are not well understood, they can lyse target cells with the perforin/granzyme system like the cytotoxic T-cells, and can secrete cytokines like IFN-γ and TNF-α upon activation. The cytotoxicity of neonatal γδ T-cells appears to be significantly less than adults [66].

T-regulatory Cells (Tregs)

T regs are a specialized subpopulation of CD4+ T cells can inhibit the proliferation of other immune cells. They exert a regulatory effect on immune cells by suppressing the proliferation of naïve T cells, the effector function of differentiated CD4 and Cd8 T cells and the function of NK cells, B cells, macrophages, osteoclasts and dendritic cells. [67]
T regs play a crucial role in establishing and maintaining self tolerance and immune homeostasis [68]; during pregnancy they´re involved in the maternal-fetal tolerance and recent studies support the concept that a normal pregnancy is associated with and elevation in the number of T reg cells.[69] Tregs appear to be elevated in preterm infants with a significant inverse correlation with GA [70].

B Lymphocytes

Pre-B-cells can be identified in the fetal liver as early as 7 weeks of gestation, and in the marrow by 12 weeks. sIgM+ B-cells are found in the fetal liver by 9 weeks and in the bone marrow, peripheral blood, and spleen by 12 weeks. B-cells with sIgA, sIgG, and sIgD isotypes appear between 10 and 12 weeks. There is also increased traffic to the lymphoid tissues, and by 22 weeks, the proportion of B-cells in the spleen, peripheral blood, and bone marrow is similar to that in adults. By 30 weeks, there are no detectable pre-B-cells in the fetal liver, and bone marrow becomes the exclusive site for B-cell maturation. Plasma cells are not generally found until 20 weeks' gestation. IgM/IgD+ B-cells populate the lymphnodes by 16–17 weeks' gestation and the spleen by 16–21 weeks. In fetal lymph nodes, primary nodules develop around the follicular dendritic cells by 17 weeks' gestation [71].
At birth, the proportion of B-cells is similar to that of adults, but the absolute number of B-cells is significantly higher [72]. The number peaks at about 3–4 months of age, and then

declines to adult levels by 6–7 years of age [73]. Preterm infants have comparable B-cell numbers to the term infants [74]. However, the number is smaller in growth-retarded infants. It has been shown that the fetus and the neonate are capable, although at a lower intensity than adults, of mounting antigen-specific antibody responses. [75,76].

It appears that the interval from birth is a more important determinant of antibody response than the gestational age. Both preterm and term infants immunized with diphtheria toxoid at 0–10 days of age had poorer responses than similarly immunized adults, but the response was better when vaccination was deferred until 1–2 months of age [77] suggesting that is the neonatal environment that plays a role in the expansion of the immune capacity [25].

Serum Ig levels remain very low until 18–20 weeks' gestation. Most of the newborn's serum immunoglobulins are derived from active transplacental transfer of maternal IgG during the third trimester [78]. For these reason while in the full-term neonate serum IgG levels are equal or even higher than maternal serum IgG levels, in the preterm the levels are lower.

After birth immunoglobulins follow a normal catabolism process and reach the lowest concentrations between 3 and 5 months of age when the infant start to produce his/her own Ig. This nadir is more pronounced and occurs earlier in preterm infants.

The serum levels of IgA, IgM and IgE are very low even in term infants, since these do not cross the placenta. However, when faced with an intrauterine infection, the fetus is definitely capable of producing appreciable amounts of IgM [79].

NK cells

NK cells can be detected as early as 6 weeks of gestation, and the number then increases progressively until birth. In cord blood, 10–15% of all lymphocytes are NK cells, which is comparable to adult peripheral blood [80]. However fetal NK cells have a significantly lower cytolytic activity against tumor cell target cell lines than adults and even at term, the cytolytic activity is only 50–80% of adult levels [81].

The Mucosal Immune System

Peyer's Patches (PP) and Other Organized Lymphoid Tissue

Peyer's patch precursors can be detected in fetal ileum at 11 weeks as aggregates of CD4+ lymphoid cells [82,83]. At birth, the organized lymphoid compartment is naive but

structurally complete, and the predominant activity involves proliferative expansion [84]. The number of PP increases from about 60 at birth to over 200 by 12–14 years [85]. Appendiceal lymphoid follicles enlarge rapidly after birth following bacterial colonization and translocation [86]. The first IgA+ plasma cells appear at 2 weeks after birth and then increase to adult levels at 4–5 months. [25]

Lymphocytes in the Lamina Propria and Intra-Epithelial Compartments

B-cells can be seen in the lamina propria at 14 weeks gestation [83]. The fetal intestinal B-cells are mainly IgM+ and IgG+ cells [87]. After birth, the IgM+ plasma cell population expands faster and at the same time microbial stimulation induces B-cells to undergo IgA class switch in both the lamina propria and organized lymphoid tissue [88]. IgA+ plasma cells are first seen in the lamina propria during the second postnatal week [89]. The number of IgA+ cells in the mucosa reach adult levels at 2 years, although serum IgA concentrations reach adult levels only during the second decade [90].

Intestinal T-cells can be identified from 12–14 weeks of gestation [91]. Several T cells precursors can be seen in the fetal intestine, suggesting that T-cells may develop locally in an extra-thymic pathway and B-cells may play a role in the development and selection of the T-cells. [83,84,91,92]

Secretory Immunoglobulins

Secretory immunoglobulins, IgA and IgM, play an important role in mucosal defense. Secretory IgA (sIgA) can be detected in mucosal secretions 1–8 weeks after birth [93,94] while sIgM appear transiently during early infancy [94].

sIgA levels rise during the neonatal period to reach an initial peak (as measured in saliva) at 4–6 weeks both in full-term and preterm infants. Despite sIgA concentrations being lower in premature infants, when chronological age is corrected for prematurity, sIgA concentrations become similar to matched full-term infants [95,96]. Salivary IgA levels continue to rise up to 18 months of age [96]. A transient nadir in sIgA has been inconsistently recorded at 3–6 months [94,97,98].

Secreted immunoglobulins also change qualitatively during the first year. There is a switch from monomeric IgA to polymeric sIgA sometime during the first year, indicating maturation of the secretory immune system [99], or alternatively, increasing exposure to exogenous

antigens [100]. The relative amounts of IgA subclasses in mucosal secretions also changes during infancy. At birth, sIgA1 is the dominant subclass but sIgA2 increases rapidly by 6 months of age [97].

During the neonatal period, colostrum provides an important alternative source of sIgA [101] and sIgA levels seem to be higher in colostrum and milk of mothers of preterm neonates [102].

Intestinal Macrophages and Dendritic Cells

Macrophages first appear in the developing intestine at 11– 12 weeks of gestation, increase rapidly between 12–22 weeks then continue to expand at a slower pace through early childhood [87,103,104]. These cells play a critical host defense role in being the first phagocytic cells of the innate immune system to encounter luminal bacteria that breach the epithelium and reach the lamina propria [105]. This is essential for sick and preterm neonates who are predisposed to bacterial translocation due to an abnormally permeable gut epithelial barrier, immaturity of the local adaptive immune system and low secretory IgA production [86,106] and therefore rely on intestinal macrophages ability to eliminate previously unknown bacteria through phagocytosis and intracellular killing.

Intestinal macrophages are derived from circulating monocytes, which are recruited to the mucosa under the influence of various epithelial and mesenchymal cell-derived chemoattractants [105,107]. Because neither intestinal macrophages nor their precursor monocytes have the ability to undergo clonal expansion [105], the only mechanism available for the development and maintenance of the gut macrophage pool is through the continuous recruitment and differentiation of blood monocytes.

3. PRETERM BIRTH AND IMMUNITY

The development of the fetal/neonatal immune system can be influenced by many perinatal factors that can either elicit maturation or induce immunosuppresion.

3.1 Antenatal glucocorticoids

Since the 1970s antenatal steroids have been administered to pregnant women at risk of preterm birth in order to induce lung maturation in the fetus and it has been demonstrated that the use of corticosteroids reduces the risk of neonatal death, respiratory distress syndrome, cerebroventricular haemorrhage, necrotising enterocolitis, infectious morbidity, need for respiratory support in preterm infants [108]. However glucocorticoids have an important role in the suppression of the immune system by acting on glucocorticoid receptors of leukocytes and thus interfering with the transcription of pro-inflammatory factors and cytokine production [109]. Even though the effects of exogenous glucocorticoids on the developing immune system have not been extensively studied in humans, animal studies suggest that glucocorticoids exposure results in long-lasting alterations to physiological and cellular responses of the offspring [110].

Studies have shown a reduction in lymphocyte number in preterm neonates after antenatal corticosteroids with an overall increase in total leukocyte count, specifically an increase in neutrophils.[111] but data on the association between antenatal steroids and increased risk of early and late onset sepsis are conflicting and not conclusive.

It has been speculated that the effects of steroids on the immune system are dependent on the levels these molecule reach in the plasma and therefore the number and the timing of steroid administration can be determinat for the immunomodulatory effect [112].

What is also unknown is whether these effects are transient or persist into childhood and beyond.

3.2 Perinatal infection and inflammation

Intrauterine inflammation is the principal cause of preterm birth [113-115]. Intrauterine inflammation can be caused by bacteria ascending from the birth canal, crossing the placenta or membranes or transferred into the amniotic cavity during amniocentesis [113,116].

Intrauterine inflammation is inversely correlated with gestational age and can be detected in up to 83% of infants weighing less than 1000 g at birth while in 10% of infants greater than 2500 g [116]. Intrauterine inflammation affects as many as 30% of all neonates born at ≤34 weeks gestation [117].

Clinical and experimental studies demonstrate that bacteria or pro-inflammatory mediators in amniotic fluid can be inspired or swallowed by the fetus to elicit a fetal inflammatory response (FIRS), characterized by an increase in fetal plasma IL-6, C-reactive protein, IL-1, IL-8, and GM-CSF [118-120]. Human studies have shown that lymphocytes are activated during infections in utero, indicating that fetal adaptive immune response is at least partly responsive [121]. Fetuses and neonates exposed to intrauterine inflammation have increased Th1 cells and increased levels of IFN-γ, indicating a potential shift from Th2 to Th1 of the fetus[6]. The shift to Th1 cytokines may lead to membrane rupture because normal term labor is partially an inflammatory event, with an increase in the production of Th1 cytokines TNF-α, IFN-γ, IL-1β, and prostaglandins in the fetal membranes and amniotic fluid [122]. Intrauterine inflammation also increases production of these cytokines [123] and prostaglandins [124]. Studies performed on animal models show that intra-amniotic lipopolysaccharide (LPS) infusion increases the number of immune cells (including monocytes, neutrophils, and lymphocytes) in fetal lung tissue. [125]

Moreover, repeated doses of LPS into the amniotic cavity of sheep, at 2 and 7 days before preterm delivery, cause a reduction in IL-6 secretion in fetal sheep when compared to a single dose of LPS showing that repeated pro-inflammatory exposures induce tolerance in preterm sheep [125]. This tolerance effect clearly demonstrates modulation of the immune system in response to the initial stimulus.

Intrauterine inflammation increases the risk of early-onset sepsis, likely because at least some of these postnatal infections originated in utero. However, intrauterine inflammation decreases the risk of late-onset sepsis [126] potentially because these infants experience immune “maturation” by the earlier (intrauterine) exposure to infection, or because they require less respiratory support and accompanying invasive care.

3.3 Mode of delivery

Differences in microbial colonization of infants at birth, as a consequence of the mode of delivery, seem to play a crucial role in the development of the immune system.

Infants delivered by caesarean section have lower diversity of gut microflora at 3 days of age than those delivered vaginally [127].

Recently it has been stressed the importance of the host microbiome for the optimal development of the individuals and it appears that caesarean section may contribute to a myriad of postnatal diseases, simply by influencing gastrointestinal colonization at birth [6].

It has also been documented that more than a half of women who deliver by cesarean section are not in labor at the time of delivery [128] and that newborn monocyte expression of TLR-2 and -4, critical mediators of innate immunity, is reduced in the absence of labor [129]. By lacking of an adequate immunological stimulus that comes from labor and vaginal delivery, newborns can therefore potentially be exposed to the risk of infection.

3.4Respiratory distress syndrome (RDS) and mechanical ventilation (MV)

Preterm infants are born before the completed development and maturation of the lung therefore virtually all the preterm infants can present with RDS: a thick blood-gas barrier, immature airway epithelium and a reduced surfactant production that lead to poor compliance, reduced ability for gas exchange and increased work of breathing and eventually need for MV [130,131]. Mechanical ventilation of the neonatal lungs can cause ventilation-induced lung injury (VILI). The shear stress, inspiratory volume, air pressure, and oxygen concentration of ventilation are believed to cause epithelial cell damage, which contributes to protein leak into the airways, inhibiting the function of surfactant and increasing inflammatory cell infiltration [130, 132,133].

The iatrogenic damange of the lung leads primarily to the activation of the innate immunity as demonstrated by the evidence of increased blood levels of neutrophils and monocytes and Cytokines such as IL-1, IL-6, IL-8 e TNF-α [130]. Neutrophils migrate thus into the airways becoming the most common inflammatory cell infiltrate [130,134].

However, adaptative immunity is also influenced by RDS and MV. It has been observed an increased number of activated T lymphocytes (expressing the CD54 marker) together with a reduced total lymphocytes count in the blood of infants with RDS [135].

It appears that lymphocytopenia of preterm infants with RDS affects primarly the CD4+ cells subset [136]. However the mechanisms that cause lymphocytopenia are not known and have been only hypothesized. A possible explanation is that activated T cells are recruited from bloodstream to the injured lungs as demonstrated by the elevated concentration of CD4+ cells

in the lung interstitium of infants with RDS [137]. Another hypothesis is that lymphocytes of infants with RDS are more likely to undergo aptoptosis phenomena compared to healthy infants as seen for lymphocytes of septic infants. Lymphocytopenia could thus be the result of an anti-inflammatory mechanism to compensate the significant immunological activation induced by lung injury.

A study performed on animal models showed that RDS and MV led to a significant inflammatory status of the lung as much as to a significant peripherical immunosuppression attested by a reduced lymphocytes responsiveness to mythogens and reduced cytokine production [138]. Another study showed that surfactant administration does not improve the immunodepression status [139]. However these data are not confirmed yet by studies perfomed in human newborns.

3.5Enteral nutrition

The intestine can be considered the largest immune organ in the body since it hosts the majority of lymphocytes and other immune cells. In newborn infants the development of a normal intestinal flora and the exposure to dietary antigens play a key role in the the generation of appropriate immune responses and the development of immune regulatory networks; however the mechanisms by which the microbes influence the phenotype and function of lymphoid cells associated with GALT are largely unknown. The Th1-Th2 balance is thought to be influenced by microbial exposure and it is likely that there is an optimum flora in early life that can promote a healthy intestine and optimize its immune function [140].

Moreover the intestine of neonates is more permeable than that of the adult and hence more susceptible to transfer and uptake of potentially harmful lumen antigens, including pathogens.

Colostrum and milk-feeding can influence the maturation of the developing intestinal epithelium and immunophenotypic differences in lymphocyte subsets following exposure to maternal milk have been reported such a decrease in CD4+:CD8+ cells ratio and an increase in IFN-γ and NK cells [141, 142].

Breast milk also contains immunosuppressive factors such as IL-10 and TGF-β that may facilitate tolerance induction to harmless food antigens and antigens associated with commensal bacteria [143].

Moreover maternal lymphocytes in human milk play an important role in modulating neonatal immune response and there are experimental evidence confirming that milk lymphocyte can

attach and traverse the neonatal intestine and can remain locally within the intestine or migrate to enter the circulation [144].

4. EVALUATION OF GLOBAL CELL-MEDIATED IMMUNITY

T-Lymphocytes, and $CD4^+$ cells in particular, play a crucial role in the regulation of the immune system. Because they´re involved in the modulation of the adaptive immunity both humoral and cell-mediated and they have also a relevant part in the control of innate immune response, they´re often targeted as a marker of the global immune function. Despite its importance and the abundance of T-lymphocyte assays that exist, the development of a standardized measurement of global cell-mediated immunity has been difficult. Typically, absolute lymphocyte counts (ALC) and $CD4^+$ T-lymphocyte counts are the only clinical assessment of immune status and immune reconstitution [145,146]. While these measurements accurately measure and track the number of T-lymphocytes, they do not reflect cell function.

4.1 Leukocytes count and lymphocyte subsets

The determination of lymphocyte population in preterm in term newborns is important for the evaluation of the immune status in a population that is particularly exposed to the risk of infection. Moreover these investigations help understanding the lymphocyte development and maturation. What is known from the studies so far published is that preterm infants' lymphocyte subsets are different from those of the term newborns and the latter have different values compared to children and adults. However most of the studies report relative instead of absolute values, which may lead to data misinterpretation. In fact, the absolute values are not affected by the relative frequencies of other subsets and constitute a more reliable indicator of the physiological immune status. [147]. The limitation of early flow cytometric technology also complicates the interpretation of data obtained in the past and the sample size of population are often either small or do not take into account preterm infants with extremely low gestational age (23-26 weeks). Juretic and colleagues [148] reported a lower percentage but a higher absolute number of T lymphocytes in cord blood compared to adult due to a higher absolute lymphocyte number in neonatal blood. While term infants showed reduced values of CD8+ cells but similar percentage of CD4+ cells compared to adults, preterm infants showed a significantly lower percentage of CD4+ to term newborns and adults. A lower percentage of T cells in infants was also documented by Peoples et al [149] and the authors found that the percentage of total T cells did not differ between term and preterm infants. Interestingly, despite the percentage of $CD4^+$ T-helper cells also being significantly lower in the combined neonatal group (term+preterm) compared with adults, only the term group's CD4+ cells were significantly lower than adults and this percentages was also

somewhat reduced compared with the preterm group. When compared with preterm infants, term infants had also a lower percentage of $CD8^+$ T cells. Both CD4+ and CD8+ memory cells were reduced in neonates compared to adults but did not different between term and preterm infants. NK cells were increased in neonates compared to adults. Conversely another study showed a reduced absolute count in lymphocyte, T cells and CD4+ subset in extremely preterm infants compared to late preterm and term infants [150]. The most elegant study was performed in 2012 on a large population of 117 preterm and 94 term infants (31-35 weeks) [147]. The authors showed that preterm infants have a low percentage of neutrophils and a high percentage of total lymphocytes with a relative high proportion of T cells (both CD4+ and CD8+) and low proportion of B and NK cells. But when they analyzed the absolute number of leukocytes they demonstrated that compared to term infants, preterms have rather a marked leukopenia involving all the subsets (lymphocyte, granulocytes, monocytes) with a significant positive correlations between these groups and GA. Treg cells were the only subset that was higher in preterm infants with a significant inverse correlation between these group and GA. These cells play a crucial role in establishing and maintaining maternal-fetal tolerance during pregnancy and the authors hypothesize that they can exert their regulatory effect by suppressing the proliferation of naïve T cells.

	n	Preterm	*n*	Term	*P* value
Total lymphocytes[a]	103	3,372 (2,274–4,516)	85	4,378 (3,507–5,957)	0.000
CD4⁺ T	102	1,605 (1,140–2,248)	81	1,942 (1,396–2,458)	0.005
CD4⁺RTEs	23	807 (538–961)	14	1,134 (821–1,937)	0.025
CD8⁺ T	102	584 (383–794)	81	739 (533–931)	0.002
CD8⁺RTEs	23	382 (318–531)	14	621 (366.2–774.8)	0.008
Tregs	29	130 (100–166)	17	146 (90–179)	NS
NK cells	103	222 (131.7–421)	78	469 (206–861)	0.000
B cells	101	518 (348–804)	77	746 (554–1,056)	0.000
Monocytes[a]	23	601 (440–804)	17	1,241 (975–1,504)	0.000
Granulocytes[a]	23	2,252 (1,222–3,959)	17	7,930 (6,491–10,142)	0.000
Basophils	23	61 (48–106)	17	130 (88–159)	0.047
Neutrophils	23	1,780 (753–3,125)	17	6,781 (5,498–8,991)	0.000
Eosinophils	23	171 (122–426)	17	346 (223–559)	0.037
Immature Grns.	23	145 (71–303)	17	577 (240–772)	0.010
Thymic function[b]					
TRECs PBMCs	33	218 (128–1,210)	13	284 (41–680)	NS
TRECs CD4⁺	17	160 (94–371)	19	154 (102–589)	NS
IL-7 (pg/ml)	85	0.50 (0.2–0.8)	68	0.95 (0.32–2.3)	0.001

Values are given as median (25th–75th percentiles). *P* value ≥ 0.05 by two-sided test was considered nonsignificant.

Grns., granulocytes; NK, natural killer; NS, nonsignificant; PBMC, peripheral blood mononuclear cell; RTEs, recent thymic emigrants; sj-TREC, signal-joint TREC; TREC, T-cell receptor gene rearrangement excision circle; Tregs, regulatory T cells.

[a]Absolute counts are shown as number of cells per μl of total cord blood. [b]TRECs values are shown as the ratio between sj-TRECs and β-TRECs.

Figure 2 Absolute count of immune cells in cord blood samples [147]

4.2 Measuring T cell immune function: Immuknow® assay

T-lymphocytes are fundamental in establishing an adequate the immune response. T-lymphocyte antigen recognition elicits a series of events such as intracellular energy production, calcium flux, phosphorylation of intracellular signal transduction proteins and ultimately de novo protein production. The characteristics and functions of the mediators produced by T-lymphocytes vary according to the context and nature of the inducing stimulation by the antigen-presenting cell, lymphoid milieu and stimulation history. The processes following T cells activation include cytokine/chemokine production, degranulation, cytotoxicity, proliferation, and eventual apoptosis or activation-induced cell death [151].

Recently, several assays have been developed in order to study T cells function ex-vivo. New flow cytometry techniques allowed to measure different intra- and extra-cellular events that follow T-cells activation: calcium flux can be detected by using fluorescently labeled calcium-sensitive dye [152]; phosphorylation induced by stimulation can be quantified by using phosphoantibodies [153]; degranulation can be measured by detecting the cell-surface expression of CD107 from cytotoxic granules [154] and ultimately cytotoxicity can be evaluated by measuring cell lysis by measuring the loss of fluorescent dye from target cells [155]. Cytokine production can also be measured by many techniques including molecular approaches for mRNA quantitation (RT-PCR or RNase protection), ELISA and ELISPOT for detection of secreted protein, and intracellular staining with flow cytometry for single-cell cytokine detection [151].

Immuknow® assay has been introduced clinically over the past several years to evaluate global cell-mediated immunity by assessing the ability of $CD4^+$ T-lymphocytes to respond to mitogenic stimulation by PHA *in vitro.* This test quantifies the amount of ATP (adenosine triphosphate) produced following overnight stimulation [156]. Immuknow® assay also requires an *in vitro* stimulation with mythogens aswell as the previously mentioned techniques, but it targets a key molecule. ATP is a basic energy source within cells, its production marks the initial step of T-lymphocyte activation and is an essential requirement for all lymphocytes functions following activation. ATP is a useful metabolic marker as it is produced within minutes to hours of initial stimulation and is necessary for cellular function regardless of eventual effector function. It can also be measured quantitatively using a

luciferin/luciferase bioluminescence system. Therefore it is a highly suitable marker for T-lymphocyte activation and a clinical evaluation of global T-lymphocyte function [151].

Immuknow® has been approved by the FDA in 2002 for the assessment T-lymphocyte function in immunosuppressed patients. The assay has been adapted to the clinical setting in its use of a small volume of whole blood, assuring that lymphocyte stimulation takes place in the presence of any drugs that may reside in the patient's system. This approach also eliminates the need for purification of mononuclear cells. In the assay, blood is diluted and added to microwells along with PHA and stimulated for 15–18 hours. The next day, the cells are isolated using magnetic particles coated with anti-CD4 antibody and then washed and lysed to liberate intracellular ATP produced in response to stimulation. ATP is quantified using a luciferin/luciferase reaction that is subsequently read in a luminometer. Light production is converted to ng/ml of ATP after extrapolation from a standard calibration curve. This standardization assures accuracy of repeat tests on the same patient over time as well as comparable results between laboratories. [151]. The process is schematic shown in figure 3.

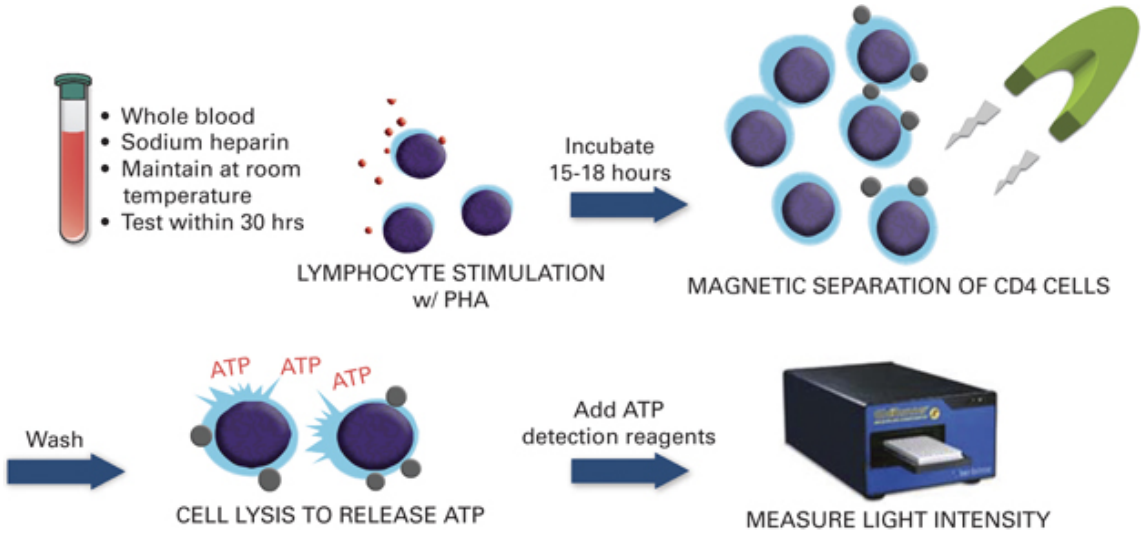


Figure 3: Immuknow® assay schematic laboratory procedure [151].

The first clinical trial was performed between 2002 and 2003 at the Univeristy of Alabama, Maryland on both healthy (n = 155) and immunosuppressed (n = 127) transplant patients by Kolwaski and colleagues [156]. The authors identified 3 zones of immune competence: ATP value (ng/ml) <225 characterized a low immune response while levels of 226–525 or > 525

indicate a moderate or strong response, respectively. These cut-off are still the conventional reference values for the evaluation of the immune response in the adults. Afterwards the same group of researchers assessed the relative risk of infection and rejection of 504 solid organ transplant recipients (heart, kidney, kidney-pancreas, liver and small bowel) using the ImmuKnow assay [157]. Blood samples were taken from recipients at established intervals after transplantion and compared with the clinical course (stable, rejection, infection). The authors reported that a recipient with an immune response value of 25 ng/ml ATP was 12 times more likely to develop an infection than a recipient with a stronger immune response and that a recipient with an immune response of 700 ng/ml ATP was 30 times more likely to develop cellular rejection than a recipient with a lower immune response value. They suggested new reference cut-off: <130 ng/mL for the risk of infection and >450 ng/mL for the risk of rejection. Since then, several studies have investigated the clinical utility of ImmuKnow. What became apparent was that a high ATP level, thought to represent under-immunosuppression, did not associate well with acute rejection events [158]. Even though the association was higher between risk for infection with very low ATP levels each study is heterogeneous from the other, differing in characteristics such as type of organ transplant, immunosuppression protocols, timing of ImmuKnow assay to event, and the measure of the event [159].

In a meta-analysis published in 2012 [160], the authors concluded that current evidence suggests that ImmuKnow assay is not able to identify individuals at risk of infection or rejection. They stated that additional studies are still needed to clarify the usefulness of this test for identifying risks of infection and rejection in transplant recipients. Literature focusing on the pediatric population is limited and there are no data on newborns both at term and preterm. In 2005 Hooper and colleagues collected samples from 50 healthy children of different ages and 37 kidney transplant recipients [161]. The mean population age was 9,1 years and only 5 children were <3 years old. Data were compared with values from adult population and the authors demonstrated that the ATP levels of children >12 years were similar to those of the adults. On the opposite, children <12 years and transplants recipients had lower values. The authors suggested new cutoffs for the pediatric population: >395 ATP ng/mL for a strong response and <175 ng/mL for a low response as reported in figure 4.

A more recent study was performed on healthy children <3 years[163]. Mean ATP levels were 376 ng/mL, in accordance to the data provided by Hooper and colleagues. As for the adults, following studies provided heterogeneous results: some authors found strong

correlations between Immuknow and both risk of infection and rejection [164] while others questioned the clinical utility of Immuknow [165].

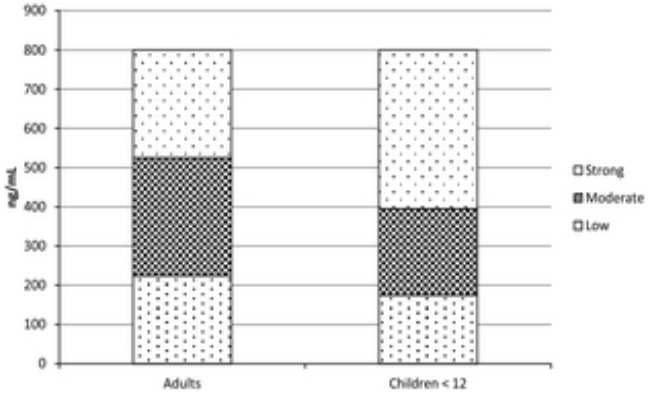


Figure 4 Immuknow® immunological zones for adults and children [162]

5. RESEARCH PROJECT

5.1 Abstract

Background. Neonatal immune system is not fully developed at birth; newborns have an adequate leukocytes and lymphocytes count at birth but these cells lack of function.
Objective. To assess the functional activity of T-cells at birth and at 30 days of life and the influence of the main perinatal factors in a population of preterm infants.
Design. A prospective longitudinal study was carried out in a population of 59 preterms. Fifteen healthy adults were included as a control group. Blood samples were collected at birth and at 30 days of life to evaluate CD4+ T cell activity using the Immuknow® assay.
Results. CD4+ T cell activity at birth and at 30 days of life were significantly lower compared with adult controls ($p < .001$). Twins showed lower activity compared to singletons ($p= .005$). Infants born to vaginal delivery had higher CD4+ T cell activity compared to those born to c-section ($p=0.031$); infants born after pPROM showed a higher activity at birth ($p= .002$). Low levels of CD4+ T cells activation at birth were associated with necrotizing enterocolitis developement in the first week of life ($p=.049$).
Conclusions. Preterm infants show a lack in CD4+ T cells activation at birth. Perinatal factors such as intrauterine inflammation, mode of delivery, zygosity can influence the levels of adaptative immune activity at birth and can contribute to expose these infants to serious complications such as NEC.

5.2 Introduction

Although immune system development begins early during fetal life, its maturation is not completed at birth, as confirmed by the increased susceptibility of newborns and preterm infants to infectious diseases.

The immune system of the fetus/newborn should protect the infant against infections at the maternal-fetal interface but should also avoid the potentially harmful pro-inflammatory/Th 1 cell-response that can induce a detrimental reaction between mother and fetus. Thus, the suppression of pro-inflammatory response helps the infant in the transition from the (sterile) intra-uterine environment to the foreign antigen-rich environment of the outside world. Therefore this inability, that has been long-time interpreted as a deficiency of the immature immune system, can actually represent a biologically advanced response.

The abnormalities of neonatal immune system are mostly related to a functional deficit of their components. Newborns and especially preterm infants have higher leukocyte and lymphocyte counts compared to adults[166]; however these cells show a lack of function at birth as a consequence of the inexperience of the adaptive immune system due to the lack of strong antigenic exposure in utero. This is confirmed by the decreased number of memory T and B cells and the increased number of naïve T and B cells into the neonatal bloodstream[167].

In oder to measure the functional activity of T-cells during the third trimester of gestation we evaluated a group of preterm infants at birth and after 30 days of life with the Immuknow® assay. This immunological test measures the level of intracellular adenosine triphosphate (ATP) after in vitro stimulation with phytohemoagglutinin (PHA) as marker of CD4+ T cells activity. It has been used in adult subjects at risk for infection, however this assay has been rarely utilized in children and never tested in newborns or preterm infants.

The aim of study was to investigate the peripheral blood CD4+ T cell activation in response to in vitro stimulation with PHA in order to assess the basal condition of the adaptive immune system at birth, its development in the first month of life and the influence of the main perinatal factors in a population of preterm infants.

5.3 Methods

Population

A prospective longitudinal study was carried out between November 2013 and July 2015 at the Neonatal Intensive Care Unit (NICU) of St. Orsola-Malpighi General Hospital in Bologna, Italy.

All the infants with gestational age (GA) $\leq$ 30 weeks and birth weight (BW) < 1500 g admitted at birth to the unit were considered elegible to the study. Infants with congenital malformations, congenital infections or born to a mother with pregnancy complications (immunosuppressive disorders, diabetes mellitus or infections during or preexisting the pregnancy) were excluded.

Before enrollment in the study, written informed consent was obtained from each infant's parents.

Fifteen healthy adults were also included as a control group.

Study design

Whole blood samples were collected in the first day of life and at 30 days of life from each patient to evaluate the pattern of lymphocytes subpopulations and the level of in vitro CD4+ T cell activity. Anamnestic and clinical data were prospectively collected during the hospital stay.

Sepsis was defined as presence of clinical signs of infection (worsening of respiratory dynamics, apnea and increased oxygen requirement, cardiovascular instability with tachycardia or bradycardia, poor perfusion, hypotonia, shock), elevation of infections markers (white cell count, CRP, procalcitonin) and a positive blood culture. Necrotizing enterocolitis (NEC) was defined according to Bell's modified criteria. [168]

The study was approved by the Sant'Orsola Hospital Research Ethics Committee (CIMPre study, 114/2012/U/Oss).

Assessment of CD4+ T cell activity

CD4+ T cell immune response was measured using the Immuknow® assay according to the package insert by the Microbiology and Virology Laboratory of Bologna University Hospital. Blood was collected in sodium heparin tubes.

Briefly, 250 µL of whole blood was diluted with sample diluent, added to wells of a 96-well microtiter plate and incubated for 15-18 h with PHA in 37°C in 5% CO_2. Magnetic particles coated with anti-human CD4 antibodies were introduced to the wells, and using a strong magnet, CD4+ T-cells were positively selected and separated. Then, the cells were lysed to release intracellular ATP. Released ATP was measured using luciferin/ luciferase and a luminometer.

CD4+ T cell immune response was defined as the quantity of intracellular ATP (ng/ml) produced after stimulation with PHA.

Statistical analysis

Statistical analysis was performed by IBM SPSS (Statistical Package for Social Sciences, version 20).

Data distribution was checked for normality by the Shapiro-Wilk test. Being data not normally distributed, non-parametric tests were used. Univariate analyses were performed in order to evaluate which clinical variables were related to ATP values at birth and at one month of life: Mann Whitney and Kruskall-Wallis test were used for categorical variables and Spearman correlation test for continuous variables. Regression analysis was performed using as independent variables all those variables which proved to be significant in the univariate analysis. Statistical significance was defined as a P value < 0.05.

5.4 Results

Seventy-three eligible infants were admitted to NICU during the study period. Fourteen infants (19,1%) were excluded because they either fulfilled the exclusion criteria (4 congenital heart disease, 4 polymalformations) or blood samples could not be collected within the first day of life (4 infants died and 2 were transferred from our unit in the first days of life).

Prenatal data and their correlations with CD4+ activity are shown in Table 1A and 1B.

	birth (59 infants)	**30 days of life (39 infants)**
Gestational age, weeks, median (range)	28 (22.7-30)	27.9 (23.3-30)
GA: 23-24 weeks, n (%)	8 (13.5)	5 (12.8)
GA: 25-27 weeks, n (%)	19 (32.2)	15 (38.5)
GA: 28-30 weeks, n (%)	32 (54.3)	19 (48.7)
Birth weight, median (range) (g)	995 (393-1500)	952 (535-1500)
Gender Male, n (%)	33 (55.9)	23 (59.0)
Singleton, n (%)	38 (64.4)	25 (64.1)
SGA n (%)	10 (16.9)	6 (15.4)
Vaginal delivery, n (%)	28 (47.5)	19 (48.7)
pPROM*, n (%)**	15 (25.4)	9 (23.0)
Prenatal steroids n(%)	48 (81.3)	33 (84.6)

Table 1A. Characteristics of study population. GA: gestational age; SGA: small for gestational age; AGA: appropriate for gestational age; ***pPROM: prolonged Premature Rupture of Membranes (> 18 hours passed between the rupture and the onset of labor/delivery).

Samples were obtained at 30 days of life from 39/59 (66,1%) of the recruited newborns, the remaining 20/59 (33,8%) encountered death or were transferred to other hospitals before 30 days of life.

	birth (59 infants)	**p value**	**30 days of life (39 infants)**	**p value**
GA: 23-24 weeks	89 (62-242)		13 (3-253)	
GA: 25-27 weeks	109.5 (44-733)		109.5 (3-383)	
GA: 28-30 weeks	85 (66-569)	p= .741	236 (57-365)	p=.029
male sex	182.9±157.1		170.5±123.0	
female sex	110.5 ±140.6	p= .091	187.1±105.3	p= .326
singleton	163 (6-733)		178 (3-383)	
twins	84 (26-153)	p= .005	168.5 (10-365)	p= .731
SGA	106.8±121.1		175.7±96.0	
AGA	164.3±158.7	p= .255	177.2±120	p= .770
vaginal delivery	123 (15-733)		123.5 (3-383)	
caesarean delivery	83.5 (6-352)	p= .031	215.5 (3-292)	p= .795
pPROM* **	197 (52-336)		185.5 (65-383)	
no pPROM* **	87 (6-733)	p= .002	168.5 (3-365)	p= .343
prenatal steroids	169.9±162.5		189.0±116.4	
no prenatal steroids	79.0±52.3	p= .140	117.0±95.4	p= .159

Table 1B. Intracellular ATP values ng/ml expressed as median (range) of the study population. ***pPROM: prolonged Premature Rupture of Membranes (>18 hours passed between the rupture and the onset of labor/delivery).

Lymphocyte subsets

The pattern of lymphocytes subpopulations at birth and at 30 days of life are reported in Table 2. While the absolute number of CD4+ did not correlate to the value of intracellular ATP at birth (p=.831) a significant positive correlation was observed at 30 days of life (p=.011).

	Birth (59 infants)	30 days (39 infants)
WBC (n)	7350 (1170-119200)	10700 (4030-34650)
N (n)	1814 (283-97744)	4267 (908-23562)
N (%)	24,8 (4,3-88)	42 (8,9-75)
L (n)	3775 (903-10775)	4141 (557-10098)
L (%)	60 (6-86,8)	40 (4,4-74)
Pan T (CD3+)	2875 (668-8404,5)	2865 (701-5481)
Pan T (%)	73,9 (47-88)	64,5 (36-84)
CD4+/ml	1954 (388-6680,5)	1982 (438-4725)
CD4+ (% L tot)	54 (32-73)	45,5 (20-69)
CD8+ml	673 (172-2312)	729,5 (198-2650,5)
CD8+ (% L tot)	18 (8-32)	16 (8-45)
CD4+/CD8+	2,92 (1,37-7,38)	2,67 (0,77-6,25)
NK/ml	242,25 (35,6-1536)	442,75 (15-1371)
NK (% L tot)	7 (1-24)	9,5 (1-42)
Pan B/ml	501 (60,56-3770)	829,35 (210-3635,3)
Pan B (%)	15 (2-30,6)	22,9 (9-41)

Table 2. Lymphocytes subpopulations at birth and at 30 days of age of infants enrolled in the study

CD4+ T cell activity in preterm newborn at birth and 30 days of age compared to adult controls

While there were no significant differences in levels of activation of CD4+ T cells at birth vs 30 days of life (median: 100 ng/ml [range: 6-733 ng/ml] vs. 168,50 ng/ml [range: 3-383 ng/ml]; p=.142), both these values were significantly lower compared with adults controls ($p < .001$) as shown in Figure 1.

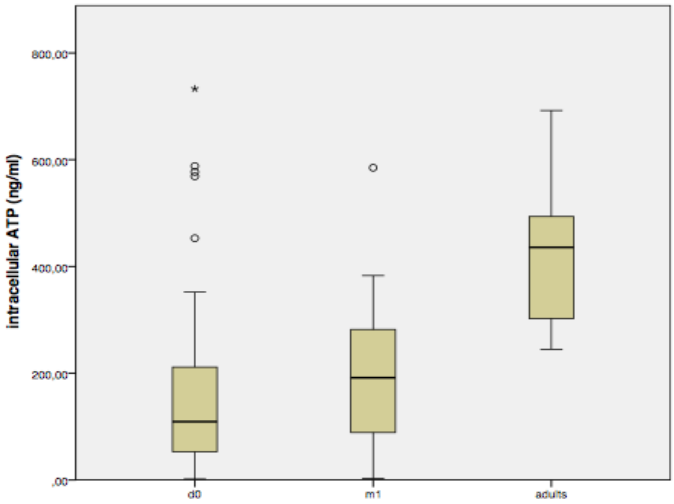
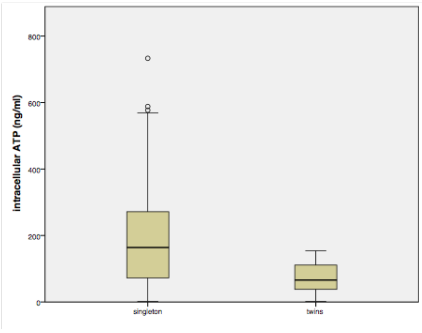


Figure 1: CD4+ T cell activity in preterm infants and in adult controls

Perinatal factors and CD4+ activity

The univariate analysis showed no significant correlation was between levels of ATP at birth and GA, BW, gender, intrauterine growth retardation (IUGR), and use of prenatal steroids (Table 1). The twenty-one twins showed significantly lower levels of intracellular ATP at birth compared to the remaining 38 singleton infants; this difference was no longer significant at 30 days of life (Table 1 and fig 2A).



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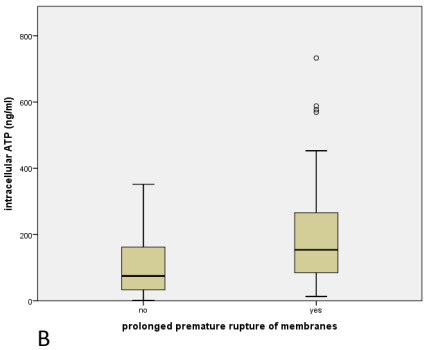

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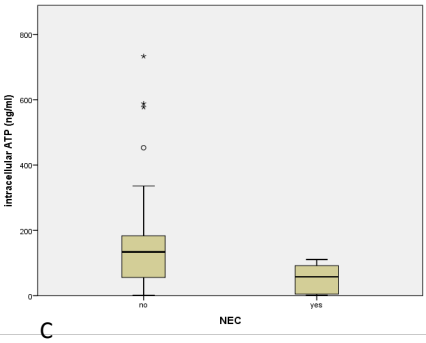

C

Figure 2. : Levels of ATP at birth. A: in twins and singletons; B: pPROM vs other conditions of delivery; C: in patients with and without NEC.

Infants born to vaginal delivery had higher levels of CD4+ activity at birth compared to those born to c-section (median 123 ng/ml vs. 83 ng/ml, p=0.031); no difference was found at 30 days of life.

Fifteen out of 59 preterm infants were born to mothers with pPROM (prolonged premature rupture of membranes > 18 hrs); these infants showed a higher activity of CD4+ T cells at birth compared to the 44 remaining infants (p= .002); this difference disappeared at 30 days of life. (Table 1 and fig 2B).

A multivariate analysis was performed including those variables which proved to be significant in the univariate analysis (pPROM and singleton/twin pregnancy): both pPROM and singleton pregnancy were independently associated with increased ATP levels at birth.

Morbidity during the first 30 days of hospital stay and CD4+ activity

A significant positive correlation was found between CD4+ activity at 30 days of life and GA (p= .029). The study population was stratified into three GA groups (23-24, 25-27, 28-30 weeks of GA) for the analysis.

While the level of intracellular ATP at birth was not different among the three groups, differences were observed at 30 days of life (Table 1). The mean levels of intracellular ATP decreased from birth to 30 days of life in the group with GA 23-24 weeks (p= .080), were similar in the group of infants born at 25-27 weeks of GA (p= 1.000) and increased significantly in the group of infants born at 28-30 weeks of GA (p= .026).

No correlation was found between CD4+ activity, both at birth and at 30 days of life, and mechanical ventilation, patency of ductus arteriosus, early onset sepsis, late onset sepsis, postnatal steroids, antibiotics use, type of enteral nutrition and death.

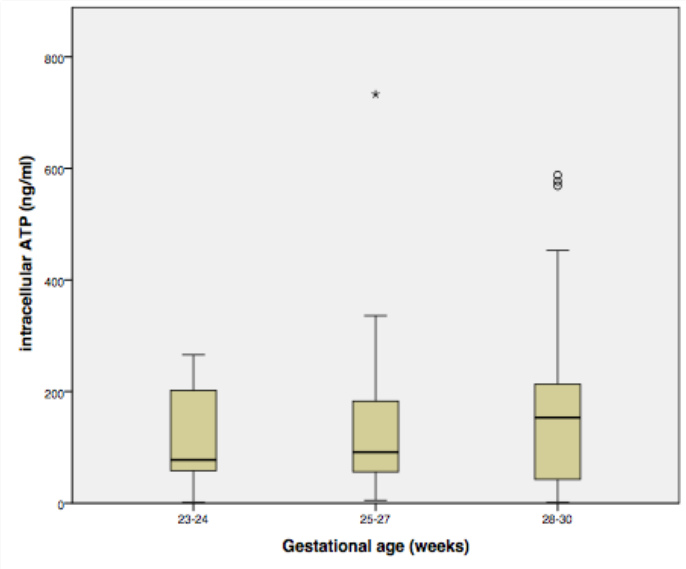

Figure 3: Levels of ATP at 30 days in different GA groups

Five out of 59 preterm infants developed NEC ≥ stage 3 during the first week of life: these infants had significantly lower values of ATP at birth compared to the 55 infants without NEC (p= .049), Fig 2C. No difference in CD4++ activity was found groups at 30 days of life .

Short term outcomes and CD4+ activity

No influence of CD4+ T cell activity on short term outcome (IVH-Intraventricular hemorrhage; PVL- periventricular leukomalacia; ROP-retinopathy of prematurity; BPD-Bronchopulmonary Dysplasia; death) was shown, both at birth and at 30 days of life.

5.5Discussion

Neonatal immune system is not fully developed at birth and newborns are therefore exposed to the risk for infection by a wide range of viruses, bacteria, protozoa, and fungi. This weakness can be partly attributed, to the lack of preexisting immunological memory and competent adaptive immunity. Newborn infants have deficiencies in T-cell activation and cytokine production, B-cell immunoglobulin production, and interactions between T and B-cells, relative to adults.[6]

T-Lymphocytes, especially CD4+ T cells, play a crucial role in the regulation of the immune system. Because they are involved in the modulation of both humoral and cell-mediated and

they have also a relevant part in the control of innate immune response, they are often targeted as a marker of the global immune function.

From 19 weeks of gestation T-cell subpopulations gradually increase in number and continue to rise after birth to peak at about 6–9 months of life. The numbers subsequently decline, and adult levels are finally reached at 6–7 years of age [43]. In term neonates, CD4+ cells constitute a higher proportion of T cells than adults. CD8+ cells, on the other hand, are fewer both in terms of their absolute number and as a percentage of total T cells. Preterm infants have a significantly higher number of CD4+ T cells while the number of CD8+ T cells does not seem to change with gestational age [44]. Reference values for T lymphocyte count are established for all ages but they do not reflect cell function.

In this study, we used the Immuknow® assay in order to investigate the function of CD4+ T cell at birth in a population of preterm infants. This test quantifies the levels of ATP after in vitro stimulation with PHA [156]. ATP is a key metabolic marker, it is produced within minutes to hours of initial stimulation and is necessary for cellular function regardless of eventual effector function and therefore it is a highly suitable marker for T-lymphocyte activation and a clinical evaluation of global T-lymphocyte function [151].

Immuknow® assay has previously only been tested in adults and children and this is the first study that uses this assay to assess T cell mediated immunity in preterm infants. This test seemed quite suitable for preterm infants since it requires a very small amount of blood to be performed (200-300 μl).

Our findings show similar leukocyte and CD4+ T cells counts to previously reported studies in preterm population [147, 150].

We documented a lack of association between the number of CD4 T cells at birth and their function (measured by intracellular ATP production) and a positive correlation between number and activity of CD4 T cells at 30 days of life. This finding underlines the concept that the absolute number of lymphocyte cells is not always an accurate estimation of the immune function. Despite being within the normal range at birth, T cells can be functionally impaired and a functional maturation can occur over time as shown in our study.

One of the peculiarities of Immuknow® assay is actually its independence of lymphocytes and CD4+ cells numbers [151,156].

In his study, Kolwalski et al. [151] demonstrated that the correlation between the number of lymphocytes the their function is weak (r= 0,24) marking the characteristic of Immuknow® assay to provide a quantification of CD4+ T cells activity that is indipendent from cells absolute number. However, other studies performed afterwards questioned this concept since a positive association between white blood cell count and ImmuKnow level was observed. [169,170].

It appears that the positive correlation between number of cells and their activity is weak when cellfunction is impaired/immature (such as in preterm infants at birth) and becomes subsequently strong when these cells encounter maturation.

We found that preterm infants have a reduced CD4+ T cell activation compared to adults: the values at birth are extremely low and, despite a trend towards higher values over time, they remain significantly low at 30 days of life. It is known that newborns, especially preterm infants, have deficiencies in both innate and adaptative immunity and many studies have demonstrated lower concentrations of cytokines such as TNF-α, IFN-α, IL-4, IL-5, IL-10, IL-15 and IFN-γ in preterms' blood compared to adults [49-52]. However cytokines production is an indirect estimation of cellular function. The present study sets the functional impairment of CD4+ T cells at the initial steps of T-lymphocyte activation, when ATP is produced. Since ATP is a basic energy source within cells, its production is an essential requirement for all lymphocytes functions following activation. [151]

While there is not a clear correlation between ATP values at birth and GA, it appears that the level of maturation of CD4+ response varies among different classes of GA. Infants born 28-30 weeks showed the expected maturational trend toward higher ATP values. On the opposite, infants born 23-24 weeks failed to develop lymphocyte activation competence and their very low ATP values at birth reached even lower values at 30 days of life. The reason of this inverted trend in unclear and this category of extremely premature infants needs to be carefully monitored .

In this study twin infants showed ATP levels at birth significantly lower compared to singletons. This is an interesting finding and literature lacks of information on the immunological peculiarities of multiple pregnancies. All the twins included in the study were dizygotic and thus immunologically different. We hypothesized that the co-presence of twins in utero may induce a deeper immune tolerance that involves both fetuses and the mother in order to avoid the potentially harmful immune reaction between the three. Our hypothesis is

in accordance with previous studies that documented higher levels of Th2-cytokines in the blood of mothers carrying twins compared with singleton pregnancies [171] underlining the more profound Th1-Th2 shift that occurs in twin pregnancies. Moreover a recent study demonstrated that both dizygotic twins and their mothers are more prone to infection than monozygotic twins, singletons and their mothers. [172]

We found that pPROM significantly increases CD4+ T cells activation at birth. It has been demonstrated that bacteria and pro-inflammatory mediators in amniotic fluid can elicit a fetal inflammatory response, documented by an increase in fetal plasma cytokines and C reactive protein [118-120]. Lymphocytes are activated during infections in utero, indicating that fetal adaptive immune response is at least partly responsive [121]. Fetuses and neonates exposed to intrauterine inflammation have increased Th1 cells response and increased levels of IFN-γ, indicating a potential shift from Th2 to Th1 of the fetus [6, 122].

We have also demonstrated that CD4+ T cells activity at birth is increased in infants born after a vaginal delivery. Increasing evidence suggests that parturition itself is an inflammatory event. [173] It has also been documented that more than a half of women who deliver by cesarean section are not in labor at the time of delivery. [128] Our findings support the current knowledge that intrauterine inflammation/infection can lead to immune maturation in the fetus. These differences disappear at 30 days of life, most likely because all the cathegories (PROM vs non-PROM and vaginal delivery vs cesarean section) are exposed to the same extrauterine enviroment.

In our study population the level of ATP at birth did not correlate with the risk of sepsis. This is not the first study that fails to detect and association between low values of ATP and infection. Previous studies performed in immunocompromised adults using Immuknow® assay showed conflicting results [159]. In a meta-analysis published in 2012 [160], the authors concluded that ImmuKnow assay is not able to identify individuals at risk of infection or rejection after organ transplant.

Significant positive correlations were observed between low levels of CD4+ T cells activation at birth and NEC development in the first week of life. Although the etiopathogenesis of NEC is still a matter of debate, some authors support the involvement of innate immune system [174]. We hypothesize that an impairment of the adaptative immune system may also play a role in the altered immune reaction that leads to NEC development. CD4+ cells impaired function may hinder infection control in the intestinal lumen. Other mechanisms

may also be involved and a more detailed characterization of CD4+ T cells is needed to clarify their role in the NEC pathogenesis.

This study has many limitations. The study sample is small and lacks of control groups both at term and with gestational age >30 weeks.

Another major limitation is the absence of a immunophenotipic classification of CD4+ T cells. The evaluation of T-reg cells is crucial to understand the immunological characteristics of the preterm infants. These CD4+ T cells are provided with immunosuppressive functions and represent a high proportion of lymphocytes at birth with a significant inverse correlation with GA [147]. The role of T-reg cells in the modulation of the immune response is an expanding field of research and this data can add precious information to our findings.

In conclusion, preterm infants show a lack in CD4+ T cells activation and fail to show a functionally maturation of lymphocyte over the first month of life. An impaired ability to respond to stimulation can contribute to expose these infants to serious complications such as NEC. However, the adaptative immune response can at least partially be elicited during the fetal life by events occurring before delivery such as pPROM or labor. Further studies in larger populations are needed to clarify these results and to better understand the cellular mechanisms that regulate neonatal adaptive immune response to pathogens.

6. REFERENCES

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