Pre-Term Exposure Patterns in Neonatal Intensive Care Unit Alters Immunological Outcome in Neonates

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Abstract

Advances in technology have lowered the limits of viability in premature births to 24 weeks of gestation. This brought forth a new population of children, who are born 3-4 months early and spent considerable amounts of time in neonatal intensive care unit (NICU), instead of sterile environment of mother’s womb. Besides, other problems associated with prematurity, these children often undergo invasive procedures resulting in mucosal inflammation and/or injury by feeding tubes, endotracheal tubes, and prolonged IV catheter. To test whether “ex-preemie-infants” were different than “term-infants” with regard to their immunity, preterm infants (< 32 weeks) and term infants (control) at the corrected age of 9-12 months were analyzed for their resting and stimulated immune responses. Preterm infants had a significant Th1 skewed response, higher number of activated and functionally competent T cells compared to term infants. The critical role of neonatal environmental exposure on immune system development is imminent; nevertheless detailed mechanistic studies on pathways are warranted.

Keywords: Neonates; Th-1 response; Th-2 response; PAMPs; Mucosal Immunity

Introduction

The prevalence of asthma and allergic disorders in developed nations has increased substantially [1]. The environmental and living conditions have been attributed to the increase in the percentage of allergic manifestations in infants and children. The “hygiene hypothesis” states that the lack of early childhood exposure to microbes increases susceptibility to allergic and infectious diseases. The priming events occurring during the early stages of the life of an infant have potential to affect the immunological outcomes in the later stages of life including developing resistance to diseases and allergy manifestations. In-utero, the immune system of the fetus is skewed towards the Th-2 type of immune response. The Th-2 type response aids in the inflammatory responses associated with allergy while the Th-1 type response is involved in fighting various microbial pathogens. Even at birth, the Th-1 response is significantly lower in magnitude compared to the Th-2 response. The Th-2 dominant immune response remains, until there is an encounter with a stimulant/ antigen, which triggers the Th-1 immune response and leads to the production of various cytokines [2,3].

Preterm infants were found to have an immune response similar to the term infants despite being born early. But term infants and preterm infants go through different kind of ‘experience’ with their immune system education during the early stages of their life. Preterm infants have stayed in Neonatal Intensive Care Unit for a long time after being born prematurely. They undergo intubation, mechanical ventilation, prolonged intravenous catheters, feeding tubes and exposure to various possible nosocomial infections in the early days of their life. This early exposure to microbial components or pathogen associated molecular patterns (PAMPs), especially the Toll-like receptor ligands may be involved in “priming” their immune system to fight against future insults. The premise for such assumption comes from previous studies [4].

In this study, we tested the hypothesis that early hospitalization of the preterm infants in Neonatal Intensive Care Units helped them ‘prime’ their immune system to have a better immune response at a later stage in their life. It was decided to test ‘resting’ and ‘stimulated’ immunity between 9-12 months of corrected age in both pre-term(subject) and term(control) infants. Preterm infants have been at home for few months and away from the effect of hospital environment. They now match closely the term infants with regard to environment as it can also affect the immune response.

The hygiene hypothesis explains the phenomenon of having a superior resistance to allergy when exposed to microbial components at an early stage in life [5]. In our previous study with murine model [6], we demonstrated that it could be extended to infectious diseases as well. Accordingly, we showed that an early exposure to PAMPs resulted in better resistance to HSV-1 infection and also helped in maintaining efficient memory. This report is novel because there is apparent lack of research focused on development aspect of immunity in preemies. Our study suggests that “ex-preemie children” are different than “term” children with regard to their immunity because of their unique experience after birth.

Materials and Methods

Study participants

The study participants were infants below the age of 12 months. These infants were apparently healthy, had no immunosuppressive...
conditions, congenital malformations or disorders and had an up-to-date immunization record. All the participating infants were born at Johnson City Medical Center (JCMC) or transferred to JCMC. The infants who were included in the study were divided into two groups. The 1st group included preterm infants with a gestation period of less than or equal to 32 weeks and were hospitalized in the NICU for over 4 weeks. The 2nd group comprised of term infants; that were born after a gestation period of 37 weeks and had no history of hospitalization in the NICU (Table 1 provides the inclusion criteria). Each participant was enrolled only after obtaining a written informed consent from the parent or the legal guardian. The study was approved by the East Tennessee state university, institutional review board.

A maximum of 5 ml blood was collected in heparin containing tubes from the infants after obtaining a detailed history from the parent/guardian. The blood was stored in two fractions, as whole blood or after isolating the peripheral blood mononuclear cells.

### Immune profile analysis

The whole blood was used to determine the lymphocytic profile of the infants. The lymphocyte profile was determined using BD Simulset™ IMK Plus kit as per the manufacturer’s instruction. The assay was performed in 96 well plates and the samples were acquired using BD FACS calibur flow cytometer and the data was analyzed using FCS express software.

### Immunocytochemistry

Peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized blood as described previously. To determine the functional ability of the CD4 and CD8 T cells, they were subjected to invitro stimulations. The stimulation was done with a cocktail of synthetic microbial components or PAMPs (pathogen associated molecular patterns) [6], consisting of palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ala-Gly-OH, polyinosinic-polyctydlic acid (poly I:C), Cytosine-phosphate-guanine containing oligodeoxyxynucleotides (CpG ODN) and Resiquimod (R848) and lipopolysaccharide (LPS). After stimulation of the PBMCs with the cocktail of PAMPs, the transport of the cytokines outside the cells was blocked using monensin (Golgistop) and incubated for 4-5 hours. Post-incubation, the cells were initially stained for the surface markers, CD4 and/or CD8. This was followed by permeabilization and intracellular staining for cytokine. The cells were stained with a cocktail of CD4, IFN-γ, IL-2 to determine Th-1 type response and CD4, IL-10 and IL-4 to evaluate the Th-2 type response. CD8 T functionality was determined after staining with a cocktail consisting of CD8, IFN-γ, TNF-α and CD107a/b. The PBMCs were incubated with Fluorescein isothiocyanate anti-human-CD107a and CD107b prior to antigen stimulation whenever analyzing the CD8 T cells cytotoxic ability. The samples were analyzed using FACS caliber 4 color flow cytometer and the data were analyzed using FCS Express software.

### Statistical analysis

The statistical analysis was performed using the graph pad prism version 4, SAS 9.1 and SPSS. Student t test was used to analyze the statistical differences between the two groups. Details are provided in a separate heading in the results section.
Results

Study population

A total of 36 infants participated in the study; of which 17 were preterm while 19 were term babies. (Table 2a) represents the details of the infants included in the two groups. All the environmental and living conditions of the participants belonging to both the groups were matched as best as possible in terms if their environmental conditions. (Table 2b).

![Figure 1: Total lymphocytic profile](image)

The figure represents the percentage of lymphocytes in the whole blood of the infants. The whole blood was subjected to evaluation using simulstest kit to obtain the % of T cell (figure 1a), B cells (fig 1b) and NK cells (fig 1c).

![Figure 2: CD4 T cell functionality](image)

*represents a p value of < 0.05.

Figure 2: CD4 T cell functionality: The figure 2a shows the percentage of CD4 T cells in the whole blood. The PBMCs were separated from the whole blood and stimulated with PAMPs and after intracellular staining (ICS), they were evaluated for their ability to produce various cytokines. Figure 2b shows the ability of the CD4 T cells to produce Th-1 cytokines. The left panel shows the percentage of CD4 T cells expressing IL-2 while the middle panel represents the % of CD4 T cells expressing IFN-$\gamma$. Polyfunctional Th-1 cytokine producing CD4 T cells are graphically represented in figure 2b, right panel. The Th-2 cytokine response is shown in figure 2c, the left and middle panel represents the IL-4 and IL-10 expressing CD4 T cells while the right panels shows the Th-2 polyfunctional (IL-4 and IL-10) CD4 T cells.
Analysis of clinical factors or complications

All infants in preterm group were born at least 28 days earlier. None of the infant in term group remained in the hospital for more than 2 days. Some of the preterm infants had to undergo procedures to address other potential health issues such as Patent Ductus Arteriosisus (PDA); Chronic Lung Disease or Broncho Pulmonary Disease (CLD/BPD); Necrotizing Enterocolitis (NEC); Sepsis with blood culture reporting positive; Intra Ventricular Hemorrhage (IVH). Besides, infants that underwent intubation, administered oxygen or put on ventilator were analyzed. The details on the number of subjects that experienced all or some of the conditions are listed in (Table 3). Statistical studies using regression analysis did not show any relationship with any factors with regard to the Th-1 and Th-2 responses.

Lymphocytic profile

To understand if early exposure to microbial components impacted the constitutive lymphocytic profile or the ‘resting’ immune response, the whole blood of the infants from both the groups were subjected to analysis to determine the frequency of T, B and NK cells in the whole blood. As shown in (Figure 1), there was no significant difference in the percentage of total T cell, B cells or NK cells. The percentage of T cells was 60.54 ± 8.88 and 63.1 ± 8.62 in the preterm in term infants respectively. 18.31 ± 6.54 was the frequency of B cells in the preterm individuals while it was 18.63 ± 7.67 in the term infants. The percentage of NK cells were higher in the preterm infants (14.61 ± 5.81) compared to the term infants (11.73 ± 4.19), but the difference between the two groups was not statistically significant.

CD4 T cell response

The peripheral blood mononuclear cells were stimulated with an optimized concentration of synthetic PAMPs to understand the ability of the CD4 T cells to produce Th-1 and Th-2 cytokines. The total frequency to CD4 T cells did not differ among the two groups (Figure 2a). However, the PAMPs induced a higher frequency of Th-1 cytokines in the preterm infants. The CD4 T cells from the preterm infants induced 14.11 ± 9.22 % of IL-2 while it was only 10.076 ± 6.68 % by the term infants (p value =0.14). The IFN-γ response was significantly higher by the CD4 T cells of the preterm infants (14.61 ± 5.81) compared to the term infants (11.73 ± 4.19), but the difference between the two groups was not statistically significant.

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exposed to microbial constituents early in life. There was approximately 10% increase in double cytokine producing CD4 T cells in the preterm groups (p value <0.001).

An inverse observation was made with respect to the Th-2 cytokines. The PBMCs were evaluated for their ability to induce Th-2 cytokines, IL-10 and IL-4 upon stimulation with various PAMPs. IL-10 cytokine response was similar in both the groups (Figure 2c). However, the IL-4 response was significantly lower in the preterm infants. The frequency of CD4+IL-4+ T cells was 9.30 ± 5.07% in preterm infants while it increased to 14.26 ± 7.6% in term infants (p value < 0.05).

**CD8 T cell response**

CD8 T cells response was analyzed after stimulation of the PBMCs with PAMPs and evaluated for their ability to produce cytokines. The total CD8 T cells frequency remained identical in both the groups (Figure 3a). However, as observed with CD4 T cells, there was a difference in the functionality of the CD8 T cells. The frequency of IFN-γ producing CD8 T cells was higher in preterm infants, though not statistically significant (Figure 3b). The response was similar in terms of TNF-α, and percentage of CD8 ‘TNFα+’ cells were statistically higher in the preterm infants (p value < 0.05). Akin to the CD4 polynfunctionally, the polynfunctional CD8 T cells were higher in preterm infant group, the frequency of CD8 T cells expressing both IFN-γ and TNF-α was significantly higher in infants who remained exposed to the hospital environment for a longer period of time (p value <0.005).

Furthermore, the cytotoxic ability measured in terms of CD107 a/b expression showed a similar elevated levels in the preterm infants. The frequency of CD107 expressing CD8 T cells were about 10% higher in preterm infants (Figure 3c).

**Statistical and power analysis**

1. Normality test: The p-values for normality test was based on the Kolmogorov- Smirnov test for actTcell, Th1 and CD8cyto are <0.01, 0.084 and 0.15, respectively. Therefore, Th1 and CD8cyto are normally distributed while actTcell is a little skewed.

2. t-test: We performed t-test for actTcell, Th1 and CD8cyto using PROC TTEST in SAS 9.1. We found that the variances between case and control groups are equal for Th1 and CD8cyto (p=0.199 and 0.839, respectively), whereas for actTcell the variances were not equal (p=0.0006). The p-values for the t-test based on equal variances for Th1 and CD8cyto are p<.0001 and 0.0014, respectively. The p-value for the t-test based on unequal variances for actTcell was 0.0116.

3. Logistic model: Based on logistic model, we found that the p-values for act Tcell, Th1 and CD8cyto are 0.029, 0.0014, and 0.0065, respectively. We concluded that the results based on t-test are similar to those using logistic regression.

4. Power analysis and sample size estimation: We used PROC POWER in SAS9.1 to calculate the statistical power and estimate the sample size. The program first specifies the type of test (two independent – samples test), two group means, two sample actual sizes, the total actual sample size, two sample standard deviations, and a levels (0.01, 0.05, and 0.10). For actTcell, if α = 0.05, the power = 0.82 . If α = 0.01, the power = 0.595. If α= 0.01, when we increase sample size up to 70, the power will be 0.94 while when we increase sample size up to 104, the power will be 0.993. For Th1, if α = 0.05, the power = 0.99 . If α = 0.01, the power = 0.98. The current sample size (N=35) has reached the 0.98 when α= 0.01. For Th1, if α = 0.05, the power = 0.925. If α = 0.01, the power = 0.773. When we increase sample size up to 105, the power will be 0.99.

**Discussion**

Our objective was to test the impact of “early hospital environment” on developing immunity. We analyzed “resting and stimulated” immune response in preterm infants at 9-12 months of life in comparison to term infants. We considered “preterm infant” who has spent at least first 28 days of life in hospital as a “different environment” compared to term infants. A total of 36 infants were enrolled in the study; 19 infants were full term and 17 infants were preterm. Both groups comprised of infants belonging to comparable environmental and living conditions. Though there was no statistical difference in the resting immune response in terms of the percentage of different immune cells, the early and prolonged hospitalization of preterm infants showed skewing towards Th-1 response suggesting possible effect of “early hospital environment” on development of immunity.

It is very well known that there is a higher prevalence of allergic disorders in developed countries when compared to developing nations [1,7]. Recently, in an elaborate experiment Figueiredo et al. [8] demonstrated difference in Th-1 cytokine profile in 1376 children from 4-11 years of age drawn from rural and urban population in Brazil. Spontaneous Cytokine production and it’s relation to environmental and biological factors [8] was highlighted. Accordingly, even in unstimulated peripheral blood cells, they found significantly more Th-1 cytokines in children from rural area compared to urban children.

In our study, patients who had history of preterm birth and hospitalization were all discharged to home environment of similar nature with term infants. So, both groups have been in “similar environment” for last 6 plus month after discharge from hospital. Characteristics of both groups with regard to exposure to other factors such as carpet, pets, smoking were similar as shown in (Table 2b). Role of indoor allergen as mentioned by Gaffin et al. [7] were not evaluated by home visit which could have some impact. [7]. But, like other environmental factor we presume, it would have effect on both groups rather than one group. Besides, effect of indoor allergen may be inconsequential in less than 1 year of age. Both groups were also similar with regard to immunomodulatory stimulation in the form of vaccination status.

Unfortunately, due to ‘chronic lung disease” attributed to prematurity related complication, preterm infants are more prone to wheezing and hospitalization secondary to severe Respiratory Syncitial Virus illness [9-11]. Hence, we do not assume that chronic lung disease associated use of nebulizer or hospital admission rate as a marker of ‘allergic disorder like asthma or reactive airway disease. There was no difference in eczema either in both groups. It is very common to have infantile eczema which could be related to food allergy to nonspecific reasons. Usually, atopic eczema continues to have prolonged course compared to other non atopic causes. However no case of eczema requiring steroid application for more than 7 days was reported in any group.

Atopy remains the main risk factor for development of asthma while indoor allergen plays casual role in disease development [7]. Asthma is the most common chronic illness of children with more prevalence in premature infants [9,10,12,13]. Also infants with more respiratory infections in the first year of life, including lower respiratory tract infections; LRTI, respiratory syncytial virus(RSV) infections or group were at higher risk of asthma at 3-10 years of age than other children [10,14-16]. Mechanism of association between LRTI, RSV and other infection may involve damage to airway epithelium in genetically predisposed individual [7,16].

The probable explanation for the difference between the two groups...
we studied may be the insults to the gastrointestinal tract (GIT). The GIT presents the largest surface area of the body to which antigens and microbes are exposed. Cause for inflammatory bowel disease in adults is believed to involve dysregulation of Th-1 and Th-2 cytokines contributing to pathogenesis [17-19]. Gut inflammation is involved in Th-1 dysregulation in patient with inflammatory bowel disease [17,18]. Necrotizing enterocolitis (NEC) in newborn is also believed to result from inflammation secondary to imbalance of pro and anti-inflammatory cytokines [20-22]. Understanding the mechanism of inflammation involved along with role of Lactobacillus has lead to novel approach of feeding formula with probiotics for prevention of NEC in preterm with some success in clinical trials [23-29]. Role of Peyer's patch in intestine has been focus of attention for local as well as systemic immunity [30,31].

Landmark papers on neonatal mice's mature immune response were instrumental in vaccination trial on human newborn [32-34]. Despite differences between adult and neonatal immune response, it is believed that under appropriate condition, protective T cell-mediated immune response could be induced by antigens in early life [2,35-45]. It is accepted by some investigators and proved that neonatal immune response is more Th-2 skewed towards immune tolerance than towards defense from microbial infection [46]. Some investigators believe that skewing in neonatal mice is more pronounced than in humans where it is diminished in magnitude [47]. But, it is shown that even in newborn, under certain stimulatory conditions involving Mycobacterium bovis bacillus Calmette-Guérin (BCG) vaccination, response can be skewed toward Th-1 phenotype [3,48]. BCG has been used and studied in attenuating atopy or allergic disease in young children in developing world by allergic questioner and PPD status [49]. Possible environment effects cannot be excluded in these studies as they were conducted in developing countries. This study may well be the first of its kind that has shown Th-1 response in infancy in a developed country like USA. However, there are reports of Th-1 response in developing countries, where it has been attributed to BCG vaccination [3]. [49] Have demonstrated Th-1 response in infancy in three developing countries where effect of environment can't be completely excluded. No solid mechanistic explanation for our observations exist as to what caused Th-1 skewed response in preterm infants, but we suggest the possibility of "hospital environment" that they were exposed to after birth along with lot of stress for survival making immune system to respond more like Th-1 rather than Th-2.

The development of a successful vaccine critically depends not only on its ability to effectively prime a response in neonates but also generate immune memory, a property that the PRR-PAMP interaction has now been shown to possess. The engagement of the TLRs (one of the PRR) with its ligand keeps the immune system alert and prepared. The exposure of newborns to microbes in the form of TLR-ligands helps skew the immune response from Th-2 to Th-1. The events leading to this involve the activation of DCs, macrophages, NK cells and Cytotoxic CD8 T cells, which leads to the upregulation of various costimulatory molecules and production of different cytokines, which are needed for fighting allergy, infection and cancer. The early immune sensitization aids in reducing the threshold needed for antigen induced T-cell activation and in turn the time taken for the adaptive immune response to get activated upon secondary stimulation with an infectious agent or a tumor antigen [50].

Much of what is known about neonatal immune system are studies done in murine models than human neonates. Compared to newborn immune status, much less has been studied of preterm infants [51,52]. There is not any study which has looked at immune status of preterm infants who had been stayed in neonatal intensive care unit and discharged home. Since, survival of premature infants has gone up in last 7-10 years, significance of learning immune response; both resting and stimulated in this population would bring new insight and fill in gap in the ontology of immune system development. The knowledge gained would help the pediatrician handling these cases to take into account the impact of environmental factors on immune response and well being.

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