Chapter 5: Results

5.1 Clinical characteristics of the subjects:

A total of 30 mothers positive for HBsAg were recruited in the study. Both peripheral and cord blood samples of 22 newborns born to HBV positive mothers were collected. Other eight HBsAg mothers, either peripheral or cord blood was not available, therefore did not counted for final results. **Newborns were divided into two groups on the basis of HBV DNA and HBsAg positivity: HBV positive (Group I: HBsAg+, HBV DNA+, N=12), HBV negative newborns (Group II: HBsAg- and HBV DNA-, N=10).** Clinical characteristics of the newborns have been described in Table 5.1.

Groups	Age (yrs ± SD)	Height (cms ± SD)	Weight (Kg ± SD)	HBV DNA (IU/ml)	HBsAg	ALT (U/L)
HBV Positive mothers (n=30)	23± 3	150 ±10	59±8	Positive 5.0x 10 (2) -10.0 x 10(7) IU/ml)	Positive	35±10
Healthy mothers (n=15)	22±4	152±10	60±8	Negative	Negative	20±10
Grp1: HBV positive Newborns (n=12)	Baseline(day0)	-	2.9±0.2	Positive 5.0x 10 (1) -10.0 x 10(4) IU/ml)	Positive	-
Grp2:HBV negative Newborns (n=10)	Baseline(day0)	-	3.2±0.2	Negative (<6 IU/ml)	Negative	-
Grp3: Healthy Newborns(n=15)	Baseline(day0)	-	3.09±0.2	Negative	Negative	-

Table 5.1

5.2 At Birth (Pre vaccination) immune responses in the newborns

5.2.1 T cell phenotypic distribution in HBsAg Positive, HBsAg Negative from HBsAg positive mothers and healthy newborns.

To investigate the differences in the immune profiles in peripheral blood of newborns, the percentage frequencies distribution of CD4+, CD8+T cells, CD45RA+naïve and CD4+CD25+FoxP3+ regulatory T cells were analyzed. There were no significant differences observed in the frequencies of CD3+CD4+T cells and CD3+CD8+T cells [27% vs. 39% vs. 21.95% P>0.05, CD3+CD4+T cells, Fig. 5.2.1(A); 55.76% vs. 58.96% vs. 78% P>0.05, Fig. 5.2.1 (B)]. In HBsAg positive newborns, significantly higher levels of T regulatory cells CD4+CD25+FOXP3+ T cells [63.79% vs. 35.22% vs. 11.06% P***<0.05, P**<0.05, Fig. 5.2.1(E)] were observed in comparison to HBsAg negative and healthy newborns. In HBsAg positive newborns, significant reduction in CD45RA+ CD45RO- naïve T cells [19.41% vs. 49% vs. 69.52%, P*< 0.05, P**<0.05, Fig.5.2.1 (D)] was observed as compared to HBsAg negative and healthy newborns.

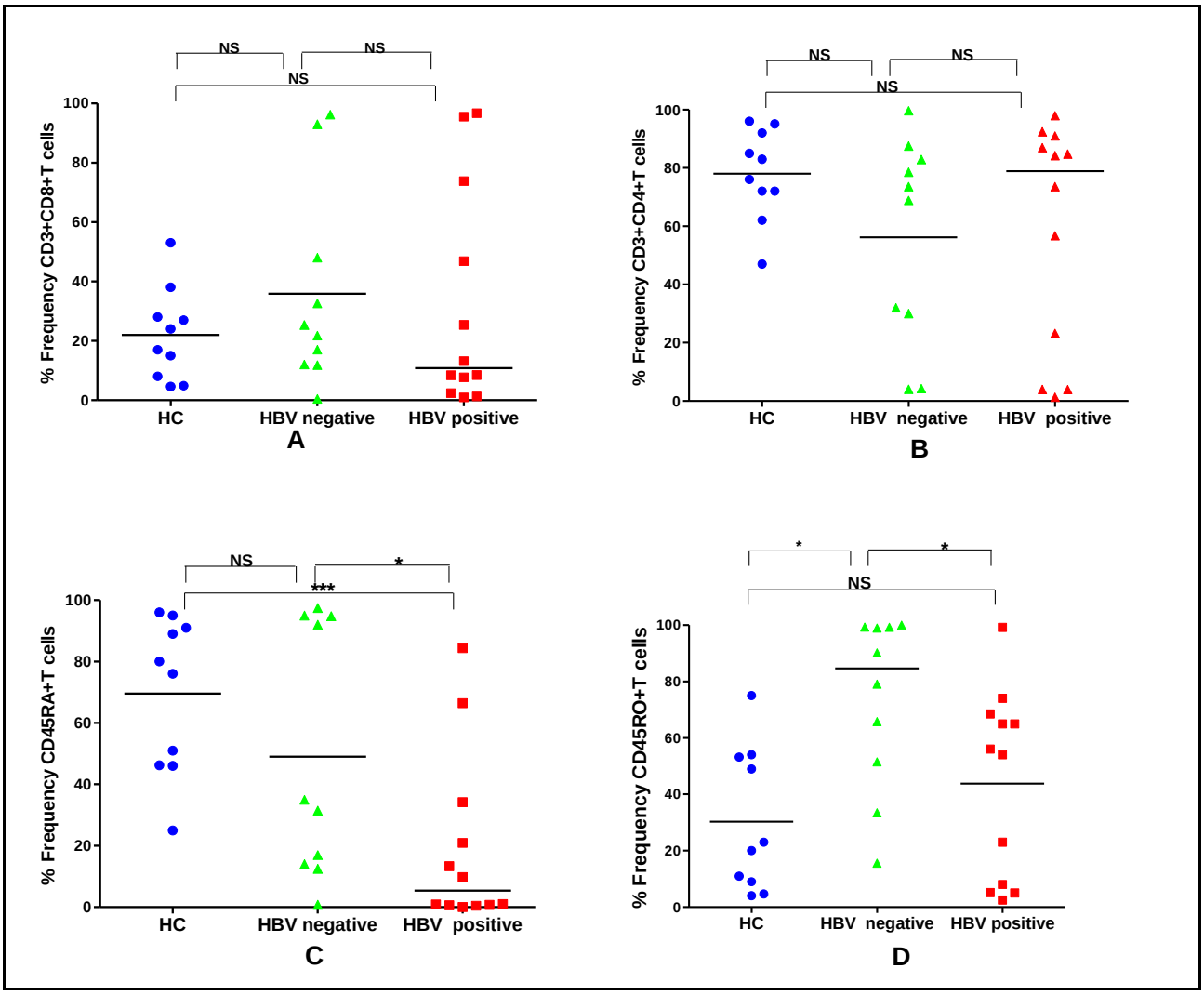

NS
NS
NS
100
80
60
40
20
0
% Frequency CD3+CD8+T cells
HC
HBV negative
HBV positive
A
% Frequency CD3+CD4+T cells
HBV negative
HBV positive
B
*
***
% Frequency CD45RA+T cells
C
% Frequency CD45RO+T cells
D

Fig. 5.2.1[A-D]:

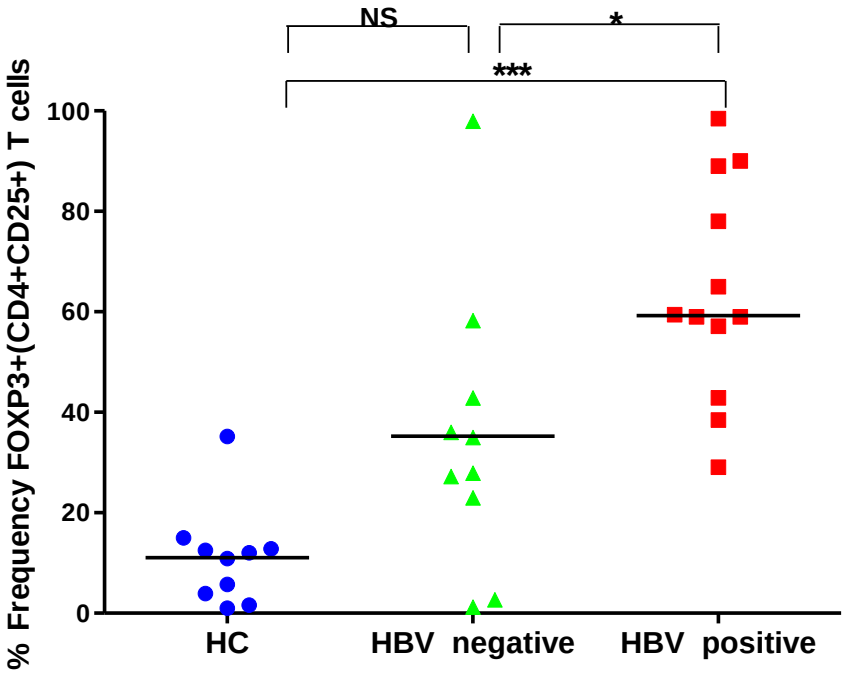


Fig. 5.2.1[E]:

5.2.2 Pre-vaccination: Lower Chemokine and Toll like receptor expression in HBsAg positive newborns:

The expression analysis of different chemokine receptors like CCR1, CCR3, CCR5, CCR6, and CCR9 on CD4 T and CD8 T cells was done. A significantly lower expression of CCR1, CCR3 and CCR5 was observed in HBsAg positive newborns compared to negative and healthy newborns [CD3+CD4+CCR1+Tcells: P*<0.05, P***<0.05; CD3+CD4+CCR3+Tcells: P*<0.05, P***<0.05; CD3+CD4+CCR5+T cells: P**<0.05, P**<0.05 (Fig 5.2.2(A-D)]. Significantly

lower expression of TLR2, TLR4 and TLR9 was observed on CD8 T cells in HBsAg positive compared to negative and healthy newborns [CD3+CD8+TLR2+T cells: P*<0.05, P**<0.05, CD3+CD8+TLR4+ T cells: P*<0.05, P**<0.05, CD3+CD8+TLR9+T cells: P*<0.05, P***<0.05 Fig 5.2.2 (E-G)].

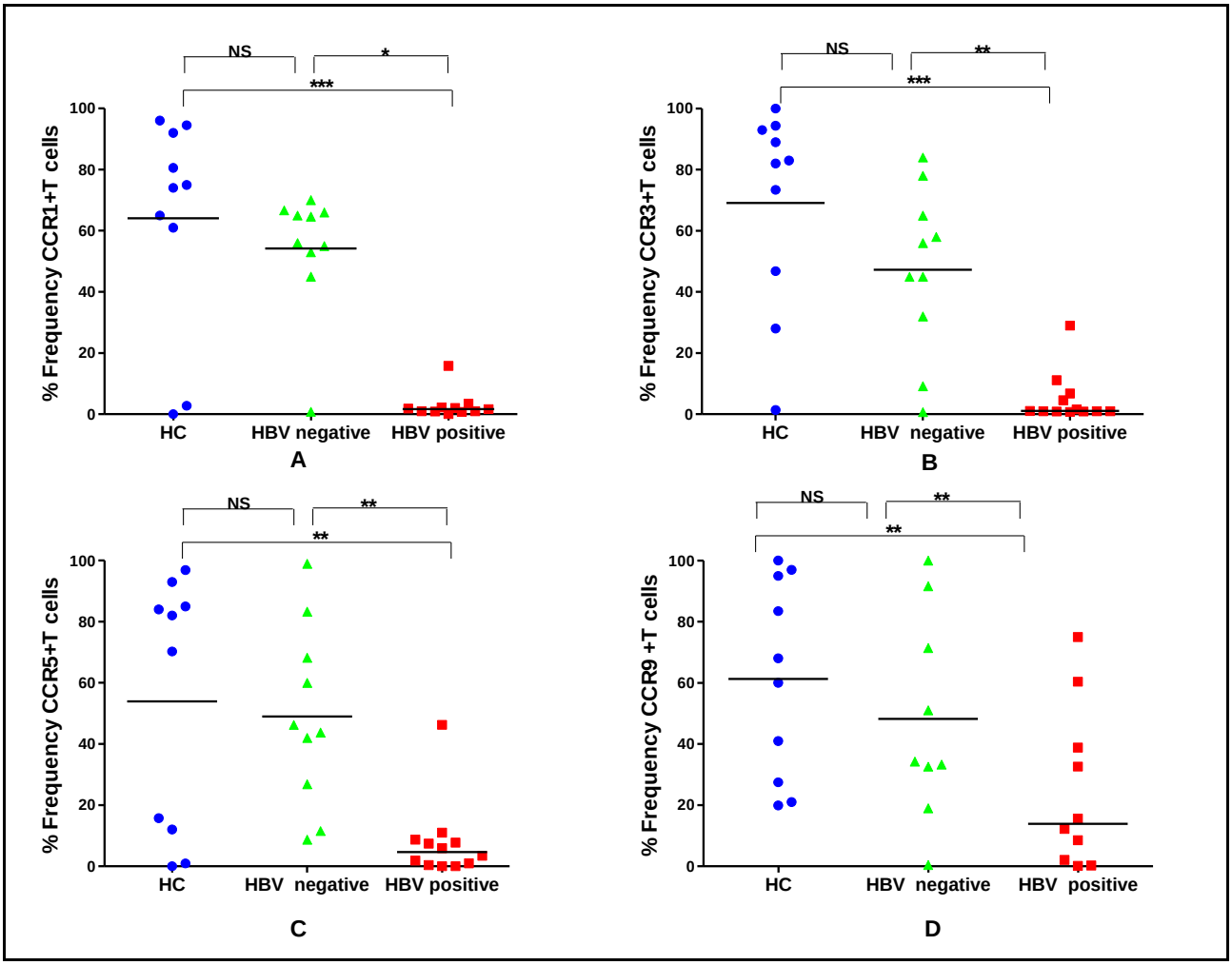


Fig. 5.2.2 (A-D)

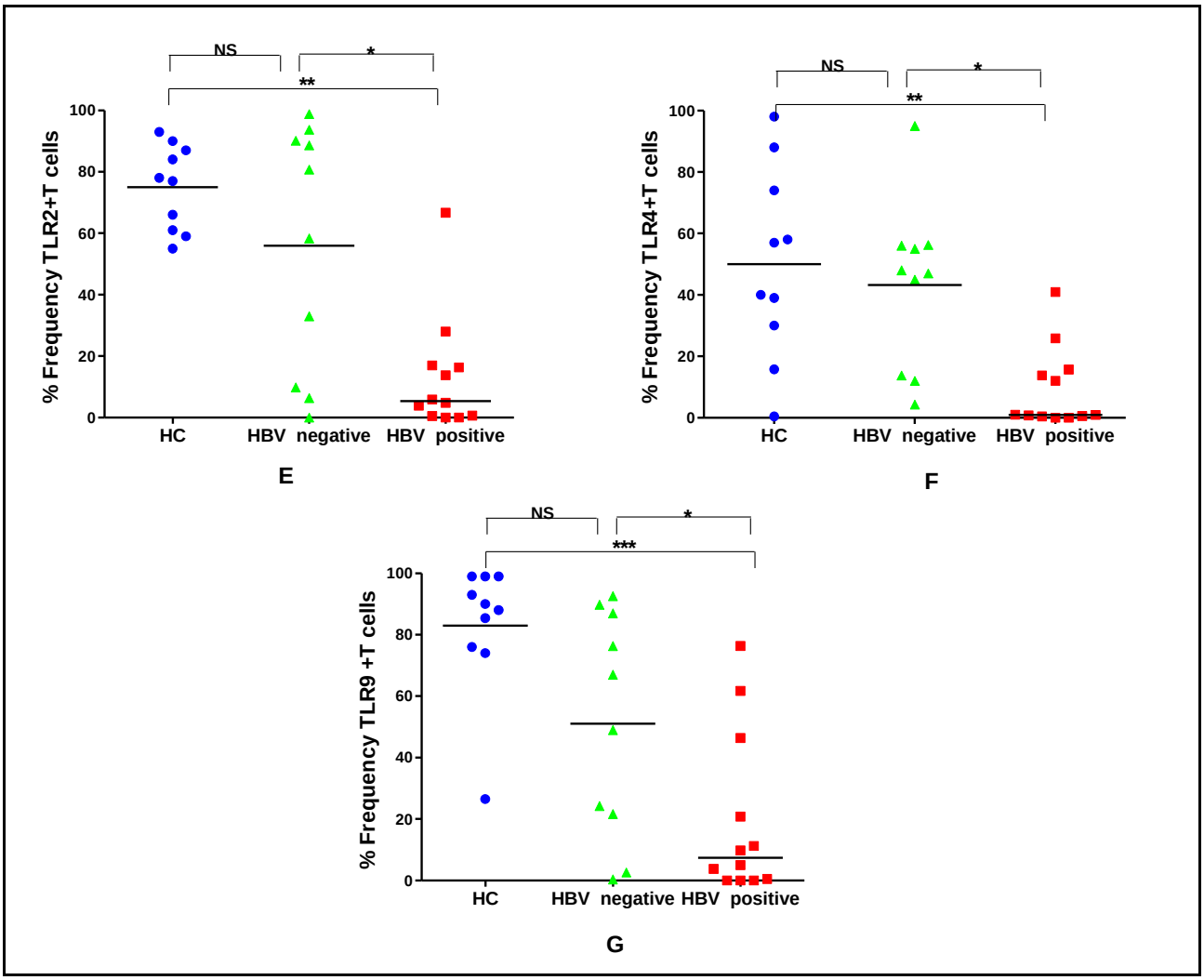
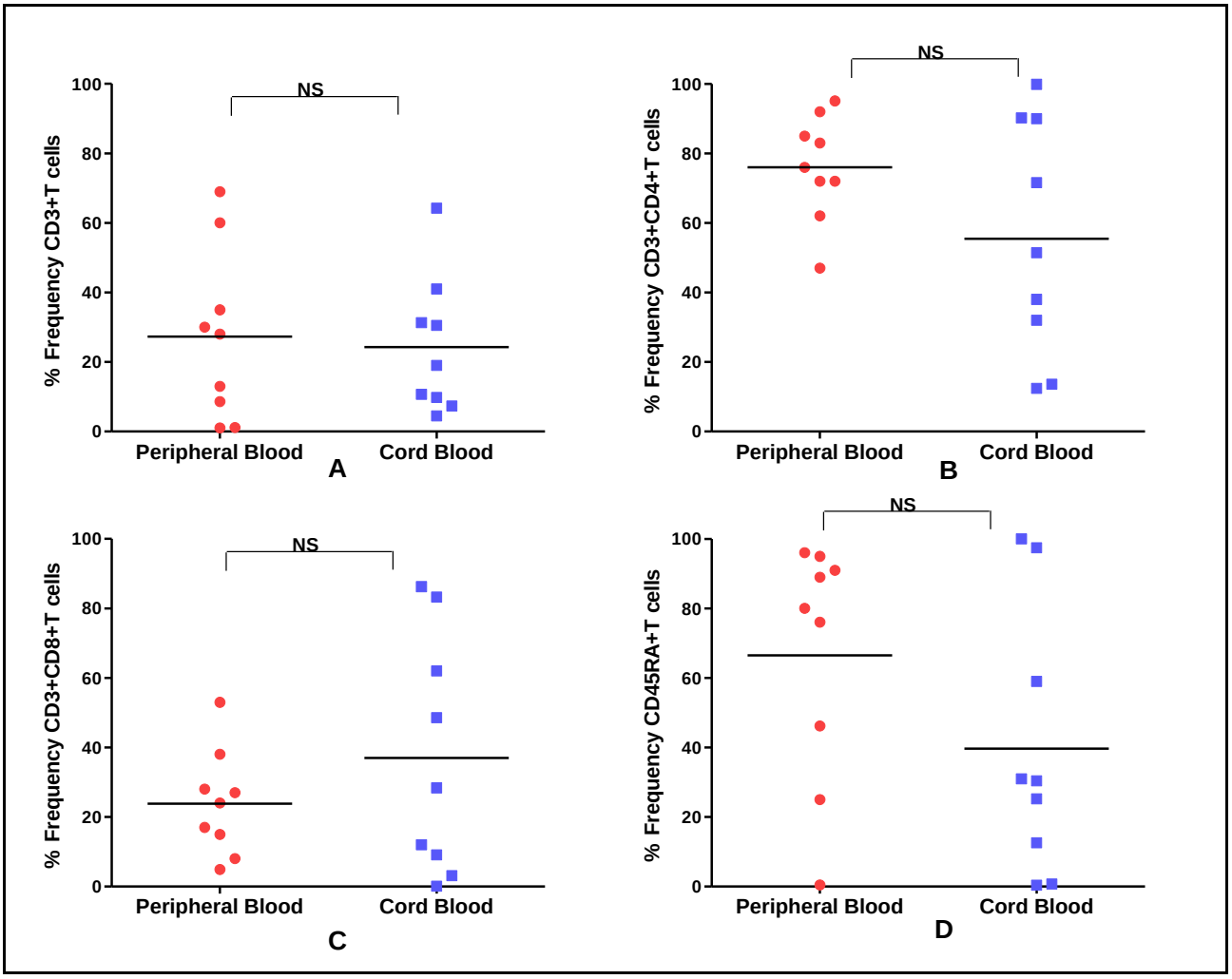

NS
*
**
% Frequency TLR2+T cells
100
80
60
40
20
0
HC
HBV negative
HBV positive
E
NS
*
**
% Frequency TLR4+T cells
100
80
60
40
20
0
HC
HBV negative
HBV positive
F
NS
*
***
% Frequency TLR9 +T cells
100
80
60
40
20
0
HC
HBV negative
HBV positive
G

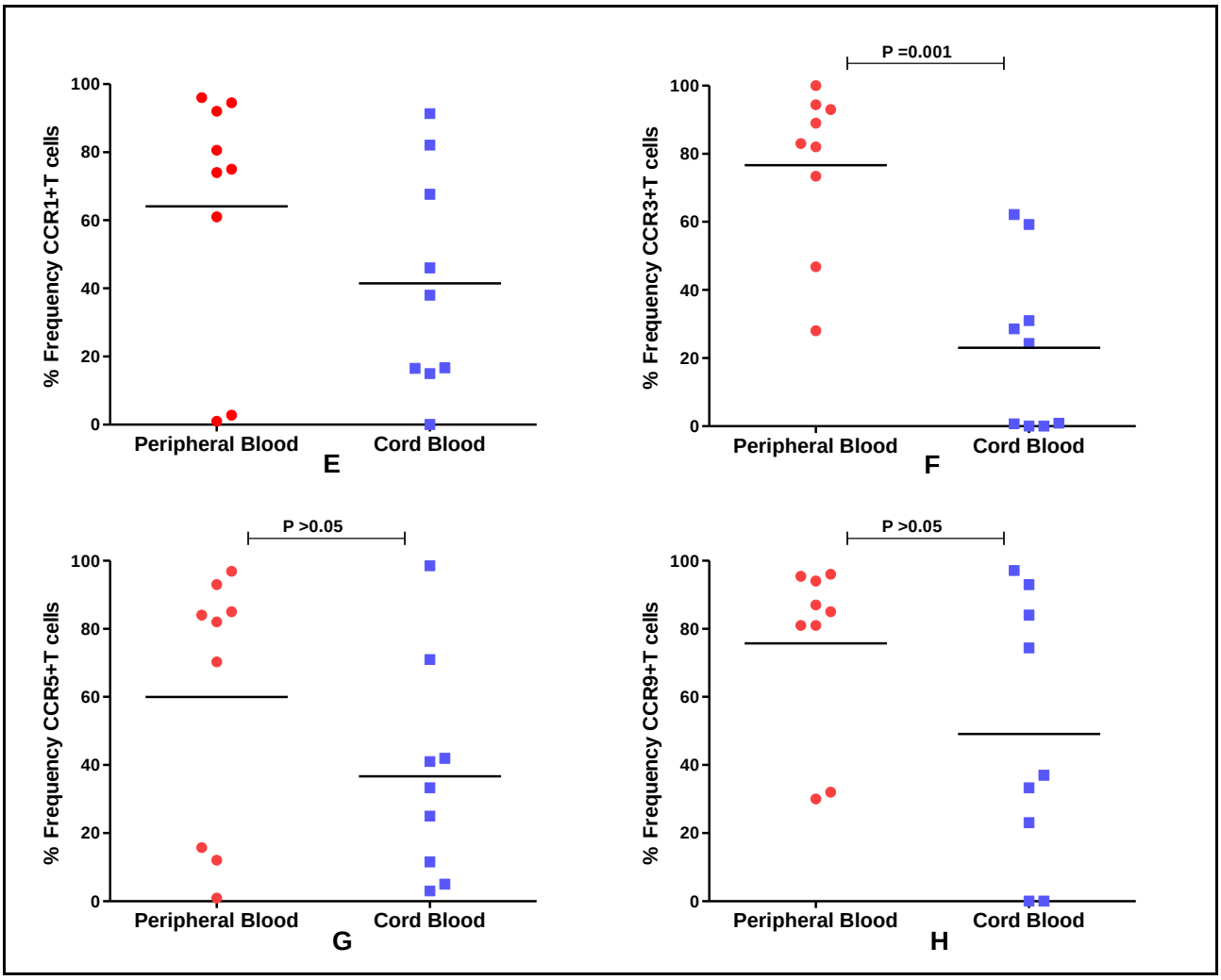
Fig. 5.2.2(E-G)

5.3 Cord Blood immune profiles of HBV positive newborns at birth

(Cord blood vs. peripheral blood)

We have compared the immune profiles in cord blood of HBsAg positive newborns and compared with the peripheral blood at birth. There were no significant differences observed in the cord blood vs. peripheral blood of newborns at birth. Therefore, we have performed the functional analysis of the T cells in cord blood, as the peripheral blood sample taken at birth was not sufficient to analyze the functional aspects.




% Frequency CCR1+T cells
100
80
60
40
20
0
Peripheral Blood
Cord Blood
E
P =0.001
% Frequency CCR3+T cells
F
P >0.05
% Frequency CCR5+T cells
G
P >0.05
% Frequency CCR9+T cells
H

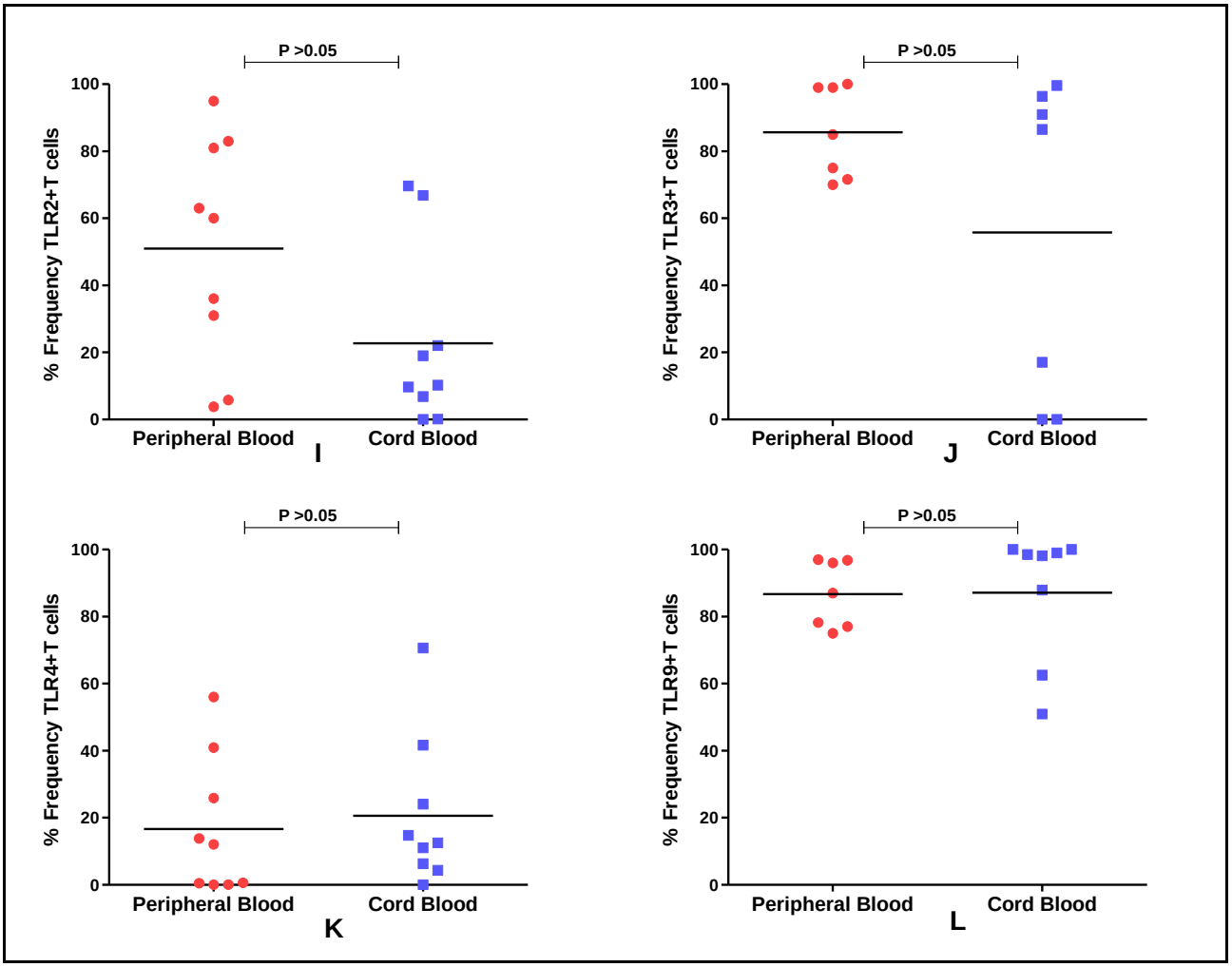
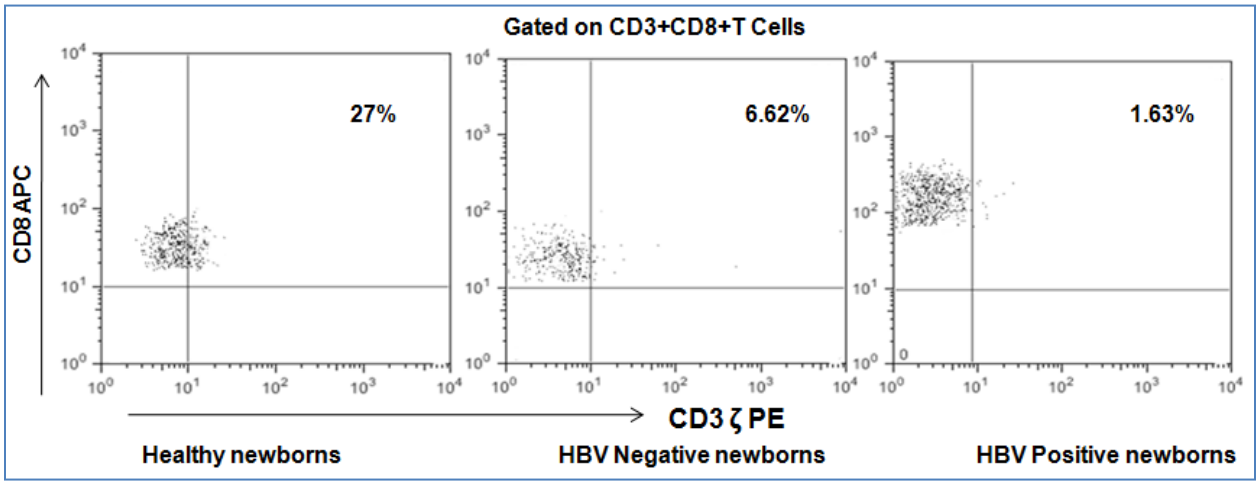

P >0.05
% Frequency TLR2+T cells
100
80
60
40
20
0
Peripheral Blood
Cord Blood
I
P >0.05
% Frequency TLR3+T cells
100
80
60
40
20
0
Peripheral Blood
Cord Blood
J
P >0.05
% Frequency TLR4+T cells
100
80
60
40
20
0
Peripheral Blood
Cord Blood
K
P >0.05
% Frequency TLR9+T cells
100
80
60
40
20
0
Peripheral Blood
Cord Blood
L

Fig.5.3 (A-L)

5.4 Phenotypic and Functional Characterization of CD8 T cells in cord blood

5.4.1 Decreased CD3 ζ chain expression on CD8 T cells in HBsAg positive newborns

To investigate the key mechanism of persistent HBV infection in newborns we have analyzed the defects lies in T cell receptor (TCRζ chain expression) and its association with the T cell dysfunction (IFNγ production and CD107A expression). We have observed significant down regulation of CD3ζ chain on CD8 T cells in positive newborns compared to HBsAg negative and healthy newborns [1.6% vs. 6.67% vs. 27.1% P**<0.05 , **[Fig.5.4.1(A)]**



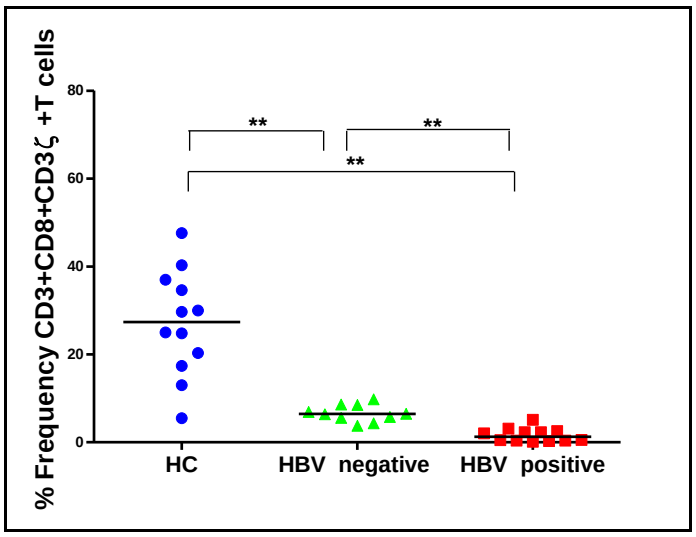


Fig.5.4.1 (A)

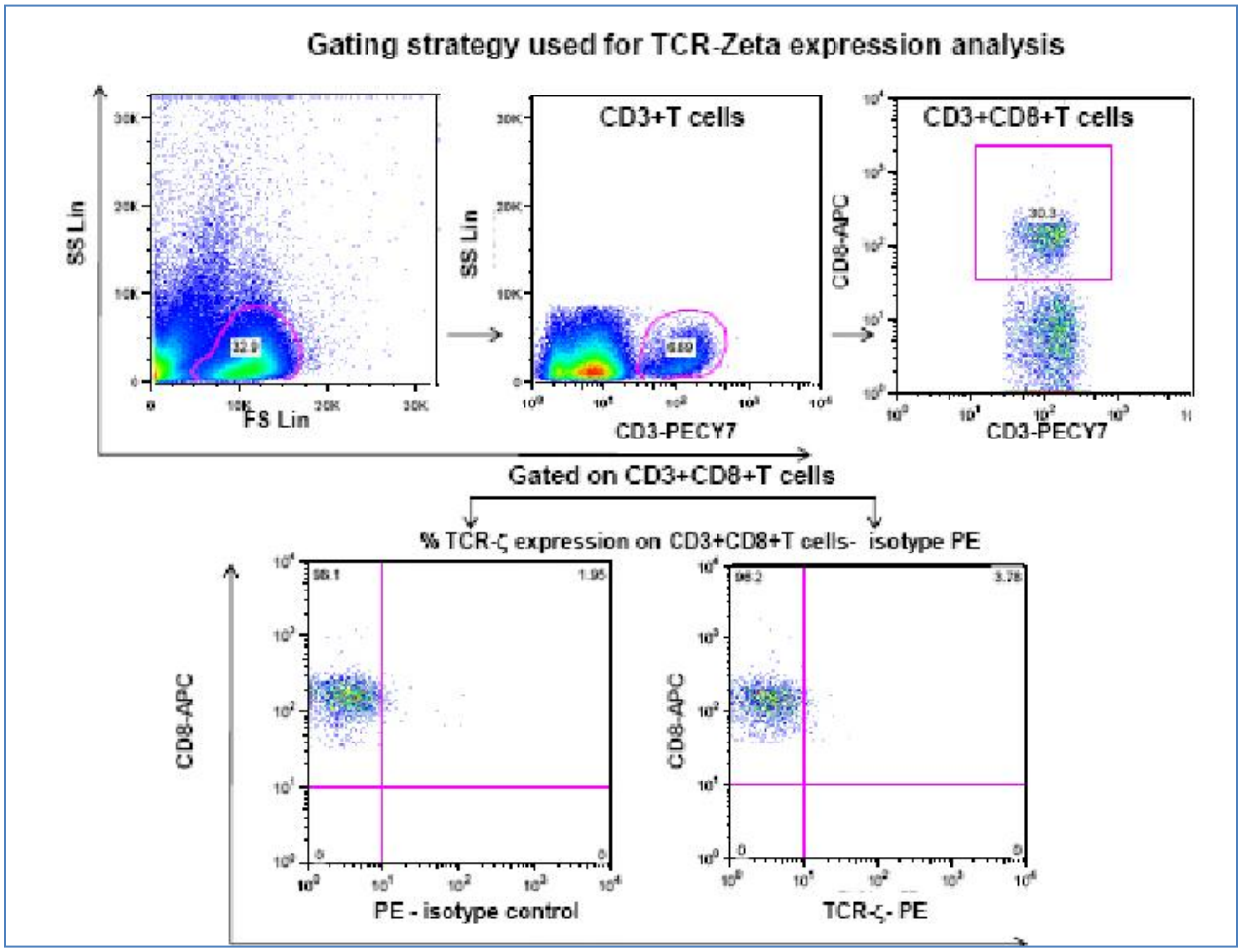
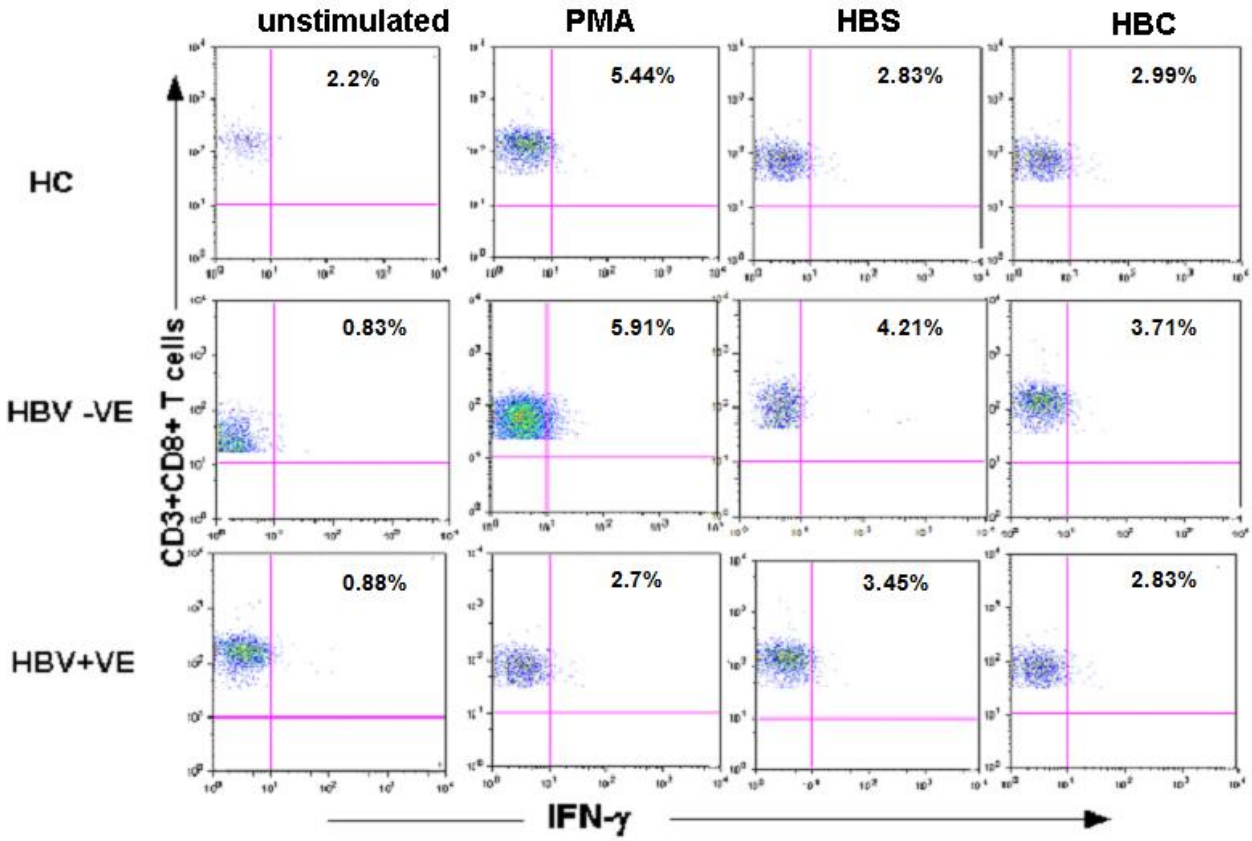


Fig.5.4.1 (B)

Fig.5.4.1 (B) Diminished expression of CD3 ζ chain on CD8 T cells in HBsAg positive newborn. Intracellular staining was done for examining CD3ζ expression on CD3+CD8+ T cells in HBV positive, negative and healthy newborns. Representative FACS and dot plots showing the expression of CD3ζ on CD3+CD8+T cells in HBsAg positive, negative and healthy newborns. CD3+ CD8+ CD3ζ+ T cells (P*<0.05, P***<0.05).

5.4.2 IFN γ production by CD8 T cells upon stimulation with PMA and viral peptides

We investigated the functional properties of CD3+CD8 T cells in HBsAg positive, negative and healthy newborns. There was down regulation observed in IFN gamma production by CD3+CD8+T cells in HBsAg positive compared to negative and healthy newborns which was significant with PMA/Ionomycin stimulation. [(Unstimulated: 0.88% vs. 0.83% vs. 2.3%, P*< 0.05; PMA: 2.7% vs. 5.9% vs. 5.4% P**<0.05; HBS: 3.45% vs. 4.21% vs. 2.83%; HBC: 2.83% vs. 3.7% vs. 2.99%, P>0.05 (Fig.5.4.2)



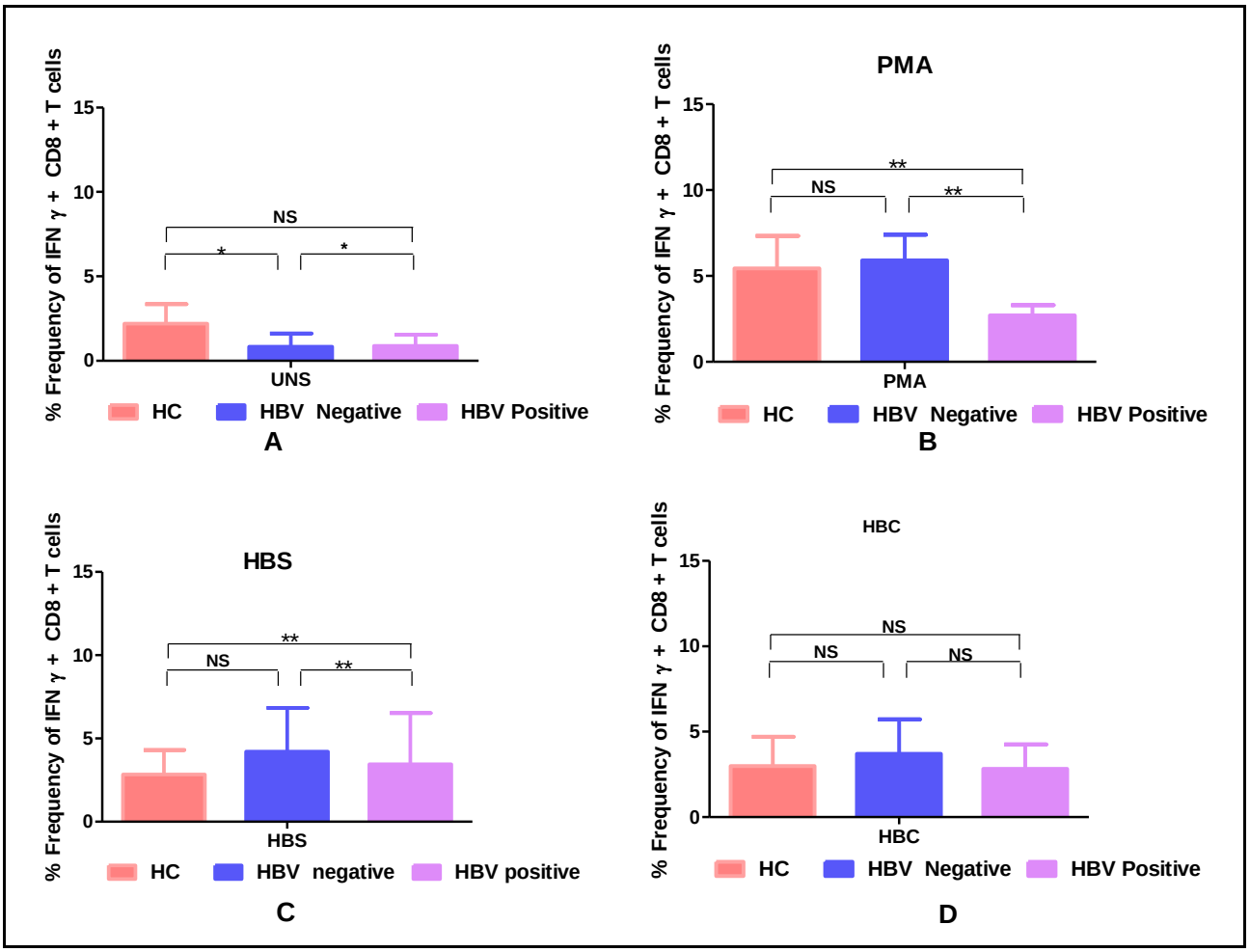
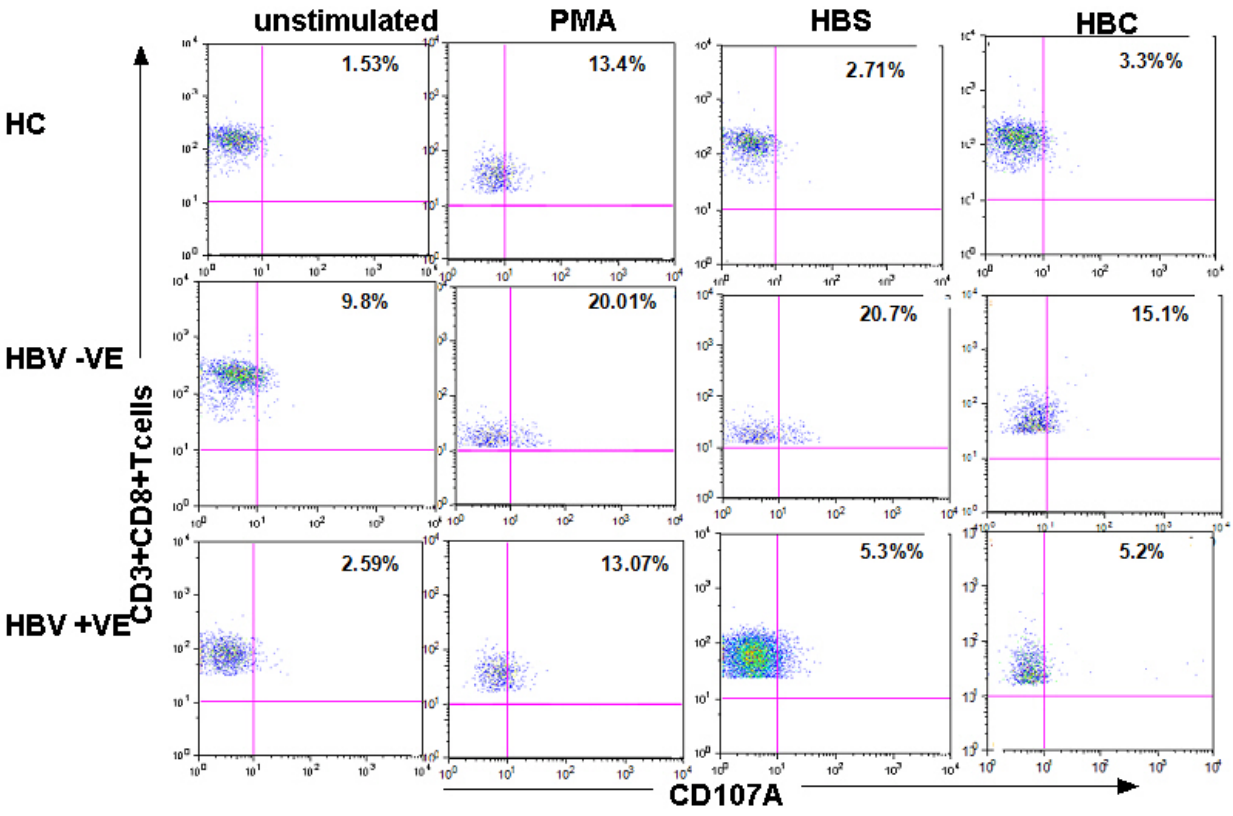


Fig. 5.4.2

Fig.5.4.2 Decreased production of IFN-γ and CD107A expression on CD8 T cells upon stimulation with PMA and viral peptides: Representative FACS and dot plots showing IFN-γ production and CD107A expression on CD3+CD8+T cells in HBsAg positive, negative and healthy newborns with and without stimulation with PMA, HBS and HBC viral surface and core specific pooled peptides for 16 hrs.

5.4.3 CD107a expression (marker of cytotoxicity)

CD107a expression on the cell surface has been described as a marker of cytotoxic CD8 T cell degranulation and was shown to be strongly up regulated on the cell surface following stimulation in concordance with loss of perforin. In HBsAg positive newborns, CD8 T cells express lesser CD107 A compared to HBsAg negative newborns with or without stimulation with PMA, HBV surface and core pooled peptides. [Unstimulated: 2.5% vs. 9.8% vs. 1.5%, P*<0.05; PMA: 13% vs. 20% vs. 13.43% P >0.05; HBS: 5.3% vs. 20.7% vs. 2.7%, P** <0.05; HBC: 5.2% vs. 15.1% vs. 2.2%, P*<0.05 [Fig.5.4.3]



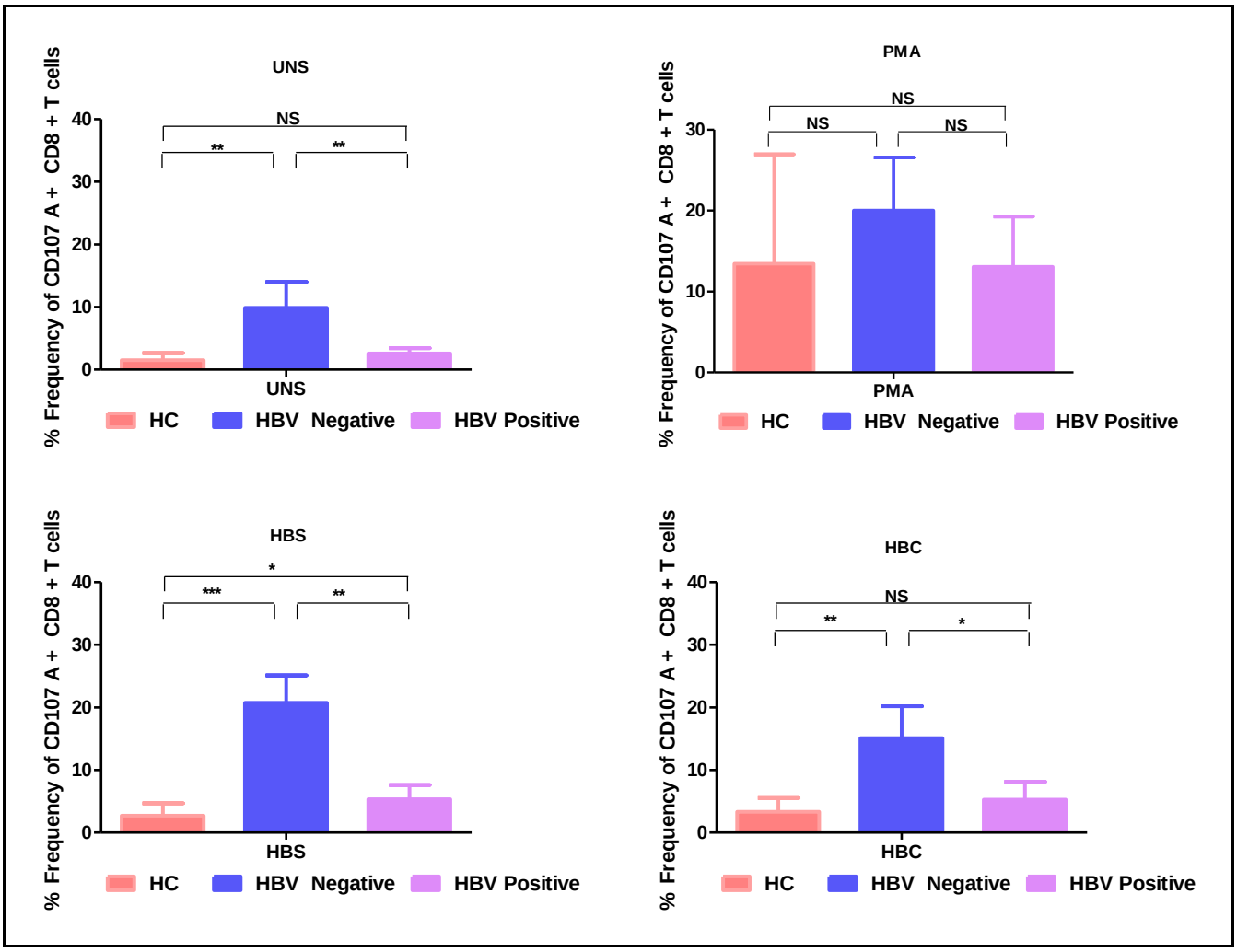


Fig.5.4.3 (A)

Fig.5.4.3(A) Decreased production of CD107A expression on CD8 T cells upon stimulation with PMA and viral peptides: Representative FACS and dot plots showing CD107A expression on CD3+CD8+T cells in HBsAg positive, negative and healthy newborns with and without stimulation with PMA, HBS and HBC viral surface and core specific pooled peptides for 16 hrs.

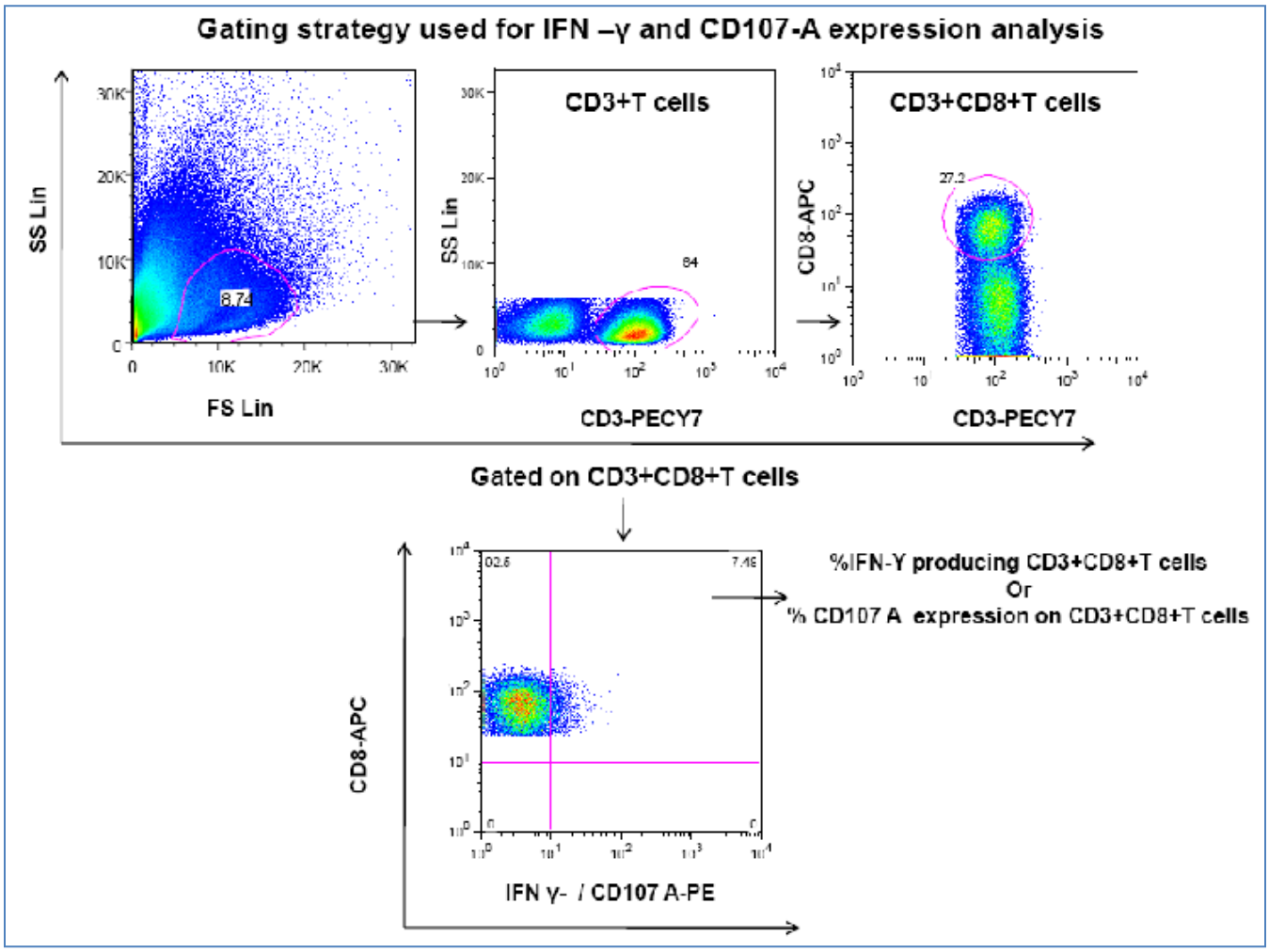


Fig.5.4.3 (B)

Fig.5.4.3(B) Gating strategy to analyze the expression of CD107A and IFN-γ on CD8 T cells

Representative FACS plots showing gating strategy for analyzing IFN-γ and CD107A expression on CD3+CD8+T cells in HBsAg positive, negative and healthy newborns with and without stimulation with PMA, HBS and HBC viral surface and core specific pooled peptides.

5.5 Post-vaccination immune responses in newborns

5.5.1 T cell frequencies (Post vaccination response)

To evaluate the efficacy of vaccination, we have assessed longitudinally the influence of vaccination on immune modulation in newborns. Immune profile in terms of percentage frequencies and statistical significance of all the immune cells studied before vaccination at baseline, and after vaccination at day14 and week 10 are given in **Table 5.1.** Compared to birth, post-vaccination, the % frequencies of CD3+CD8+T cells (P>0.05, P>0.05) and CD3+CD4+T cells were increased (P>0.05, P>0.05), though the differences were not significant. The frequencies of CD8+CD69+ activated T cells significantly increased from baseline at post-vaccination compared to healthy newborns (P*<0.05, P*<0.05) [Fig. 5.5.1(A-E]. Despite vaccination no changes were observed in the T regulatory cells frequencies, CD4+CD25+FoxP3+ (P>0.05, P>0.05) in HBV positive newborns.

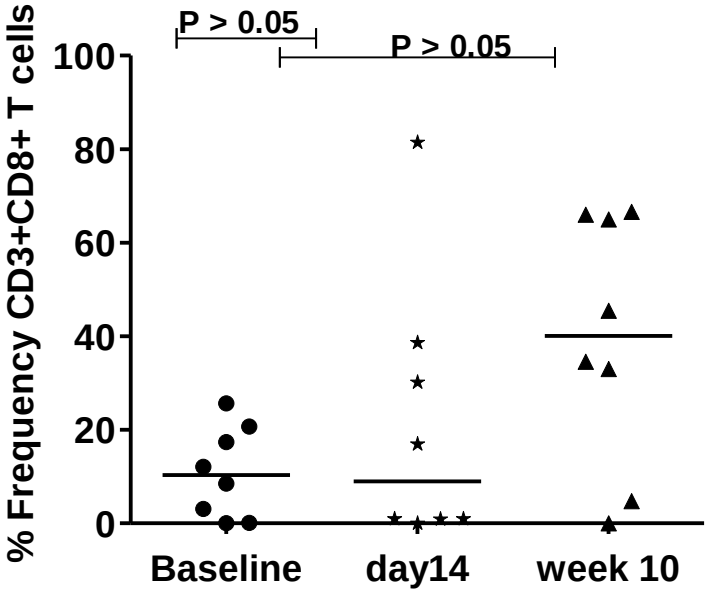


Fig.5.5.1 (A)

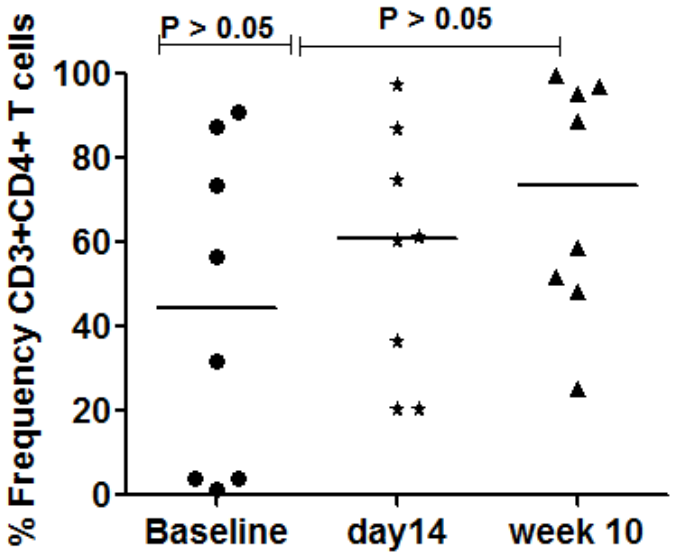


Fig.5.5.1 (B)

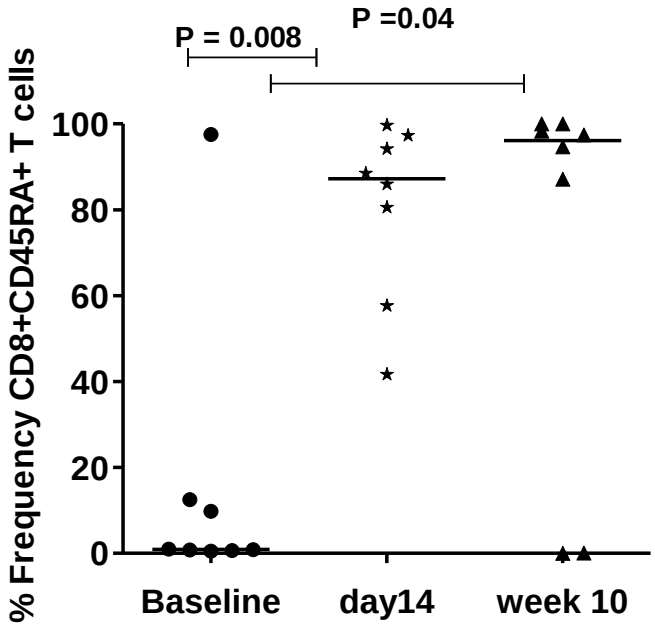


Fig.5.5.1(C)

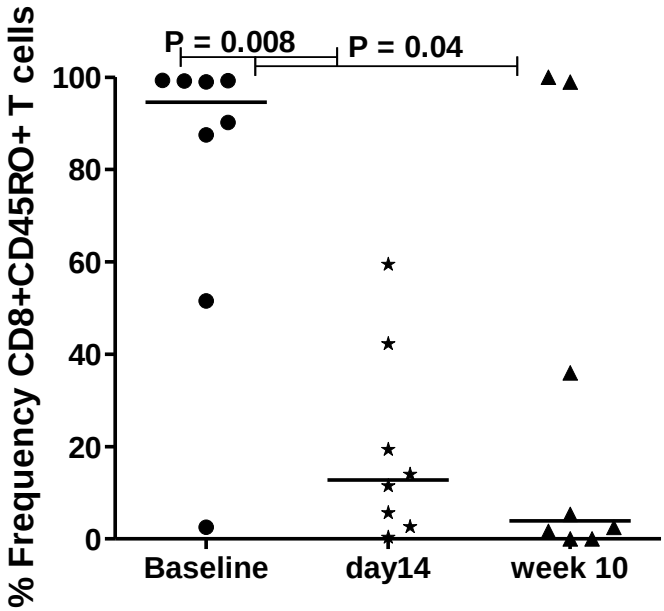


Fig. 5.5.1(D)

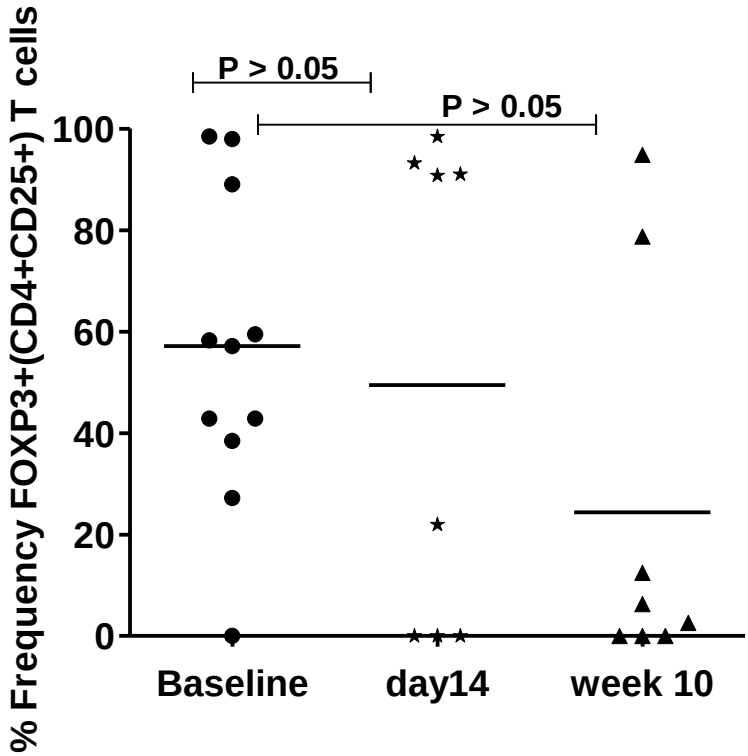


Fig. 5.5.1(E)

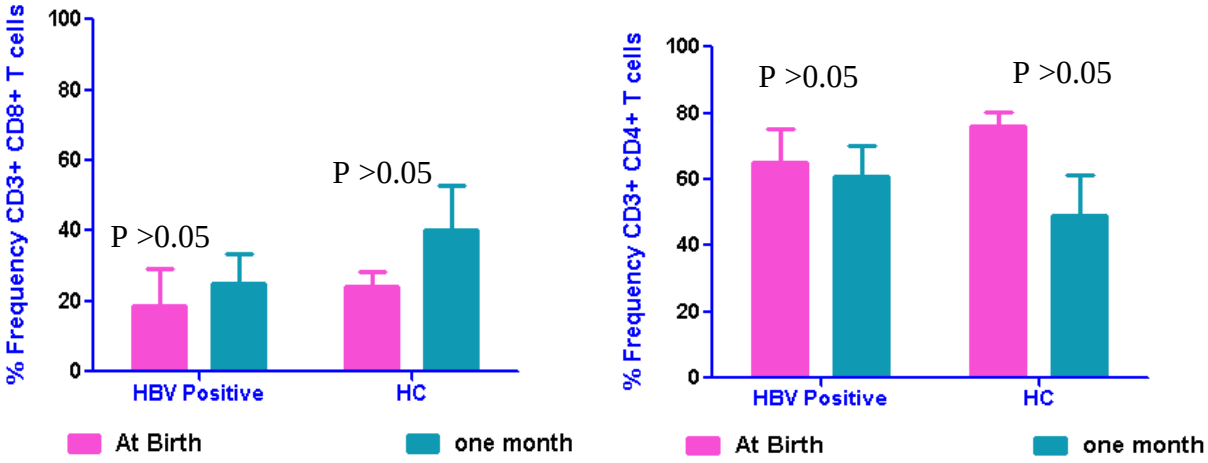


Fig. 5.5.1(F) No significant differences in CD4 and CD8 T cells

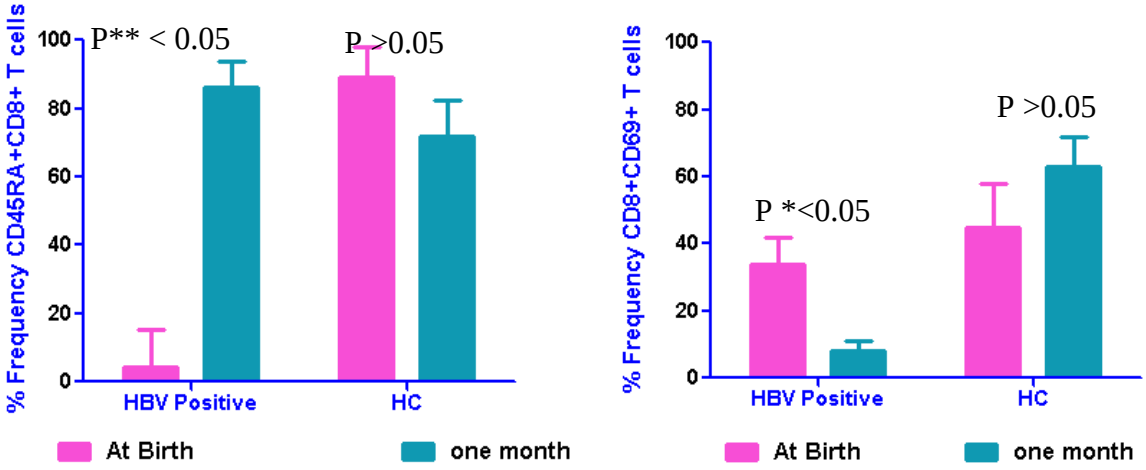


Fig. 5.5.1(G) Increased Naïve T cells and decreased activated T cells in HBsAg positive newborns

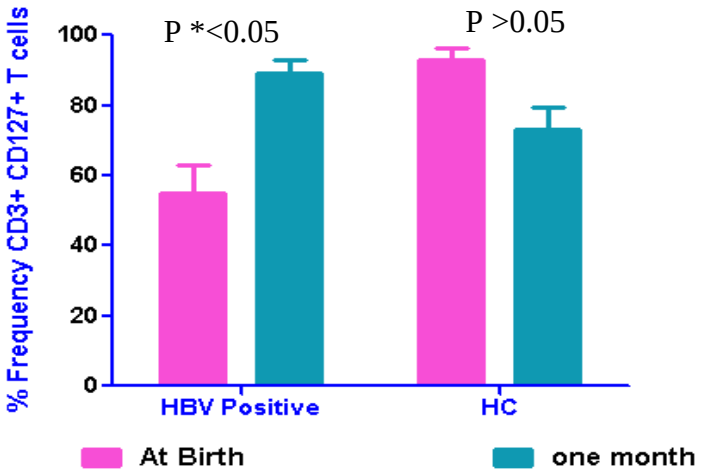


Fig. 5.5.1(H) Increased expression of Memory T cells

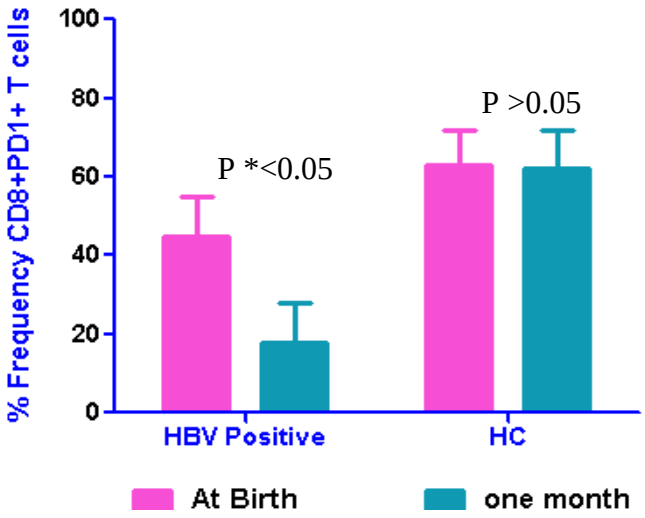


Fig. 5.5.1(I) Decreased expression of PD1 on CD8 T cells in HBsAg positive newborns.

5.5.2 Vaccination improved the Chemokine receptor CCR1, CCR3, CCR9 and Toll like receptor TLR2, TLR4 and TLR9 expression in HBsAg positive newborns compared to healthy newborns.

The baseline immune response before vaccination and at day 14 and week 10 post-vaccination were studied in the newborns. Vaccination significantly enhanced the expression of CCR1, CCR3, CCR5 and CCR9 on T cells, 0 day vs. day14 vs. week 10; CD4+CCR1+T cells: 0.83% vs. 93.54% vs. 93.88%, P**<0.05, P>0.05) ; CD4+CCR3+ T cells : 0.42% vs. 88.53% vs. 89% and P**< 0.05, P*<0.05); CD4+CCR9+T cells (33.79% vs. 92.02%, 77.65 P*<0.05, P*<0.05 [Fig.5.5.2]. Vaccination significantly increased the expression of TLR2, TLR4 and TLR9 on T cells, 0 day vs. day14 vs. week 10 [CD8+TLR2+ T cells 69.53% vs. 89.81% vs. 85.41%, P*<0.05, P>0.05; CD8+TLR4+T cells; 49.42% vs. 85.09% vs. 83.09%, P>0.05, P>0.05, CD8+TLR9+ T cells; 88.83% vs. 98.39% vs.77.67%, P*<0.05, P>0.05 [Fig.5.5.2 C]. In HBsAg p ositive newborns rapid increase in naïve T cells, Chemokine and Toll like receptor expression was observed at one month post vaccination in comparison to healthy newborns in which there was gradual age dependent increase.

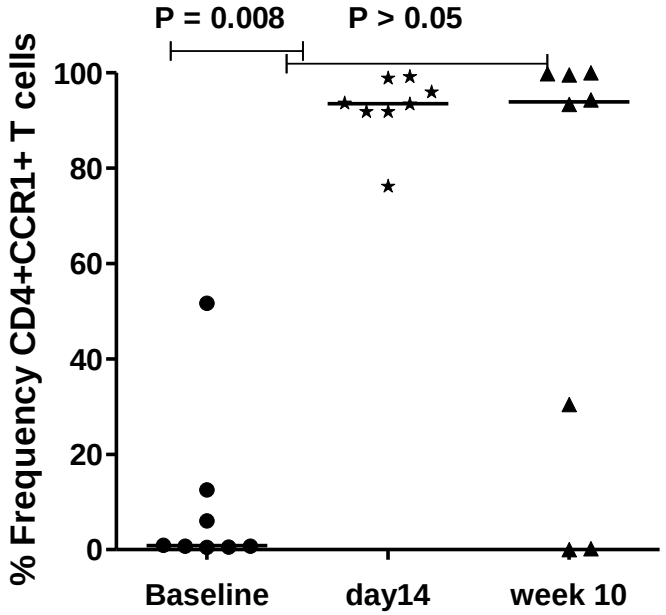


Fig. 5.5.2(A)

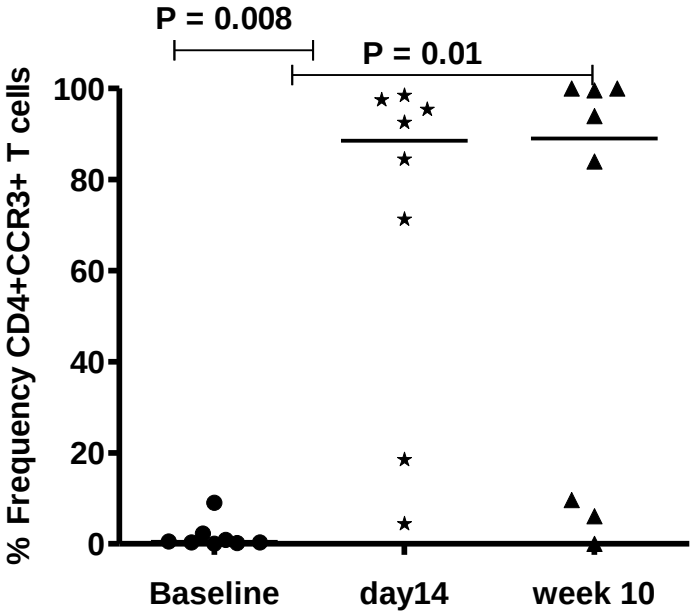


Fig. 5.5.2(B)

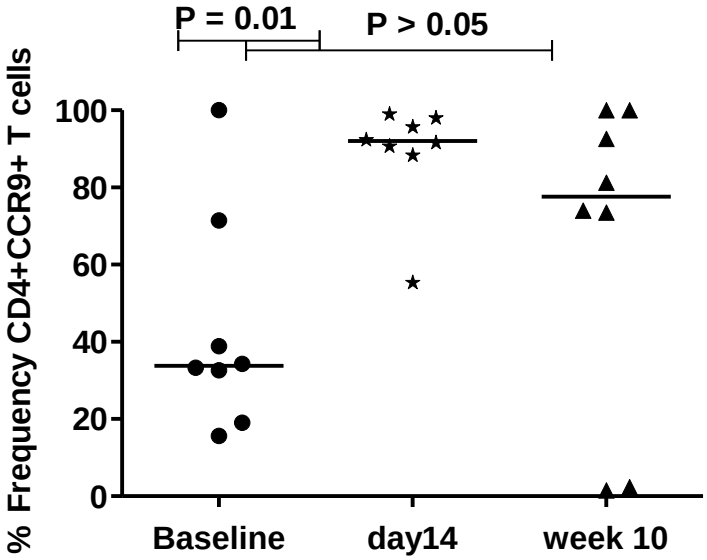


Fig. 5.5.2(C)

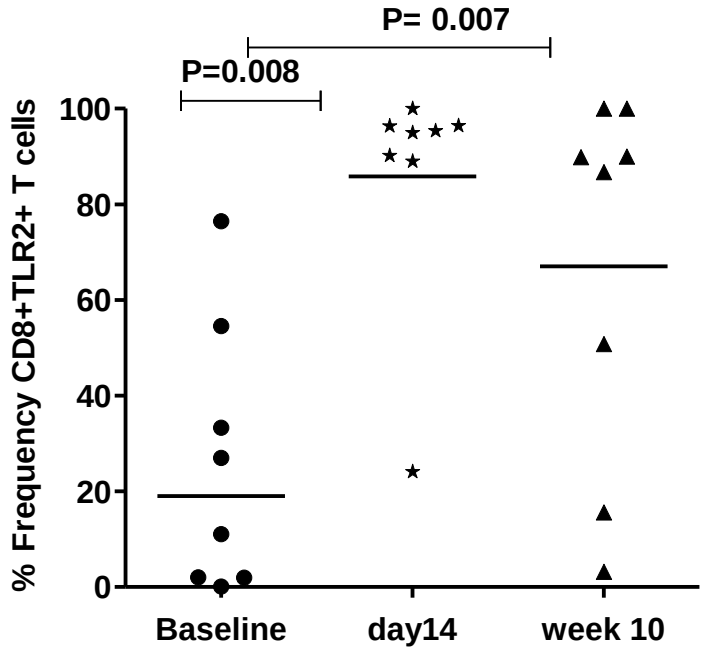


Fig. 5.5.2(D)

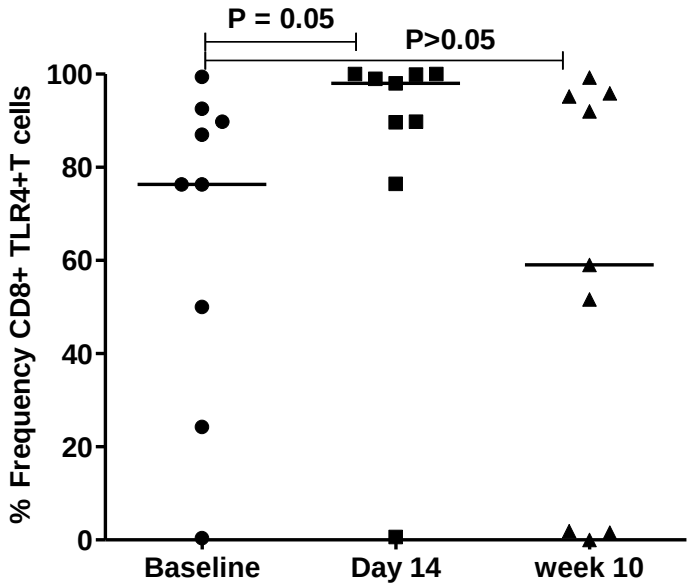


Fig. 5.5.2(E)

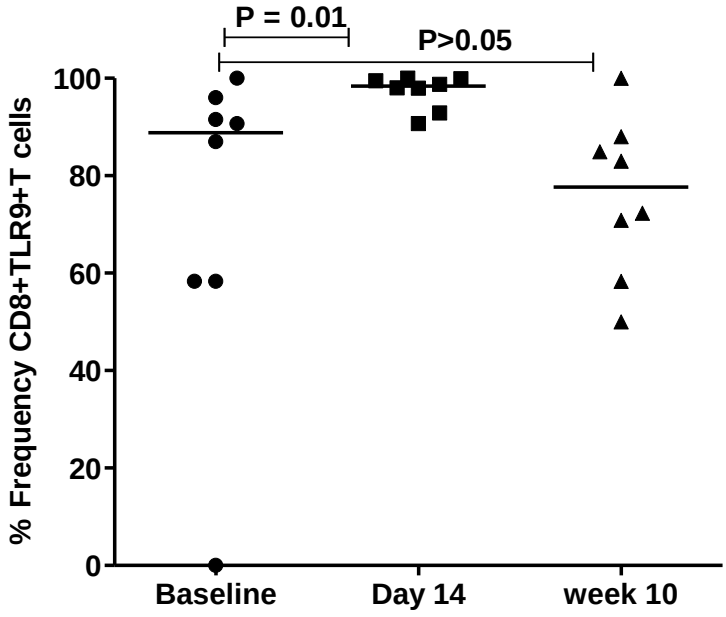


Fig. 5.5.2(F)

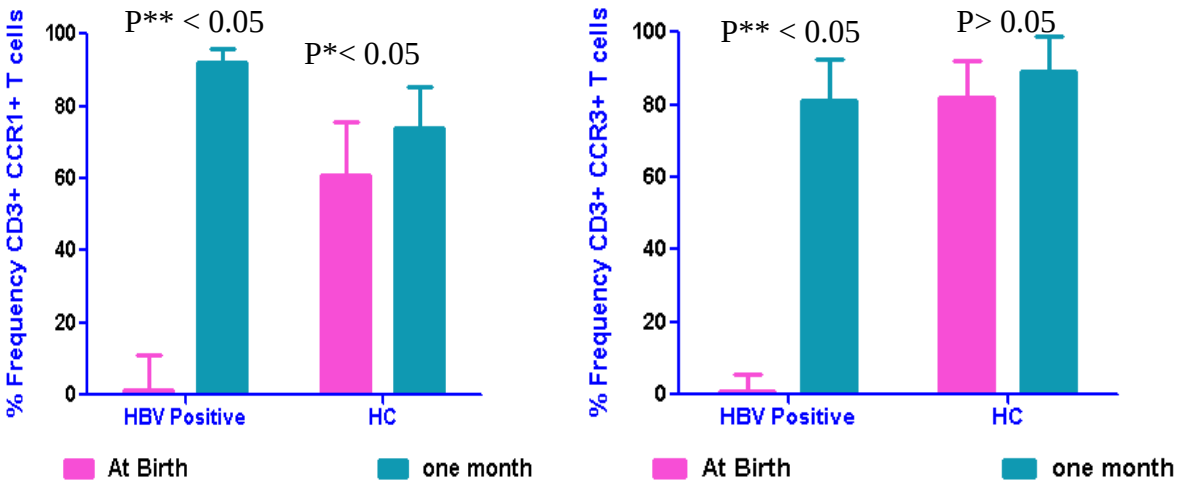
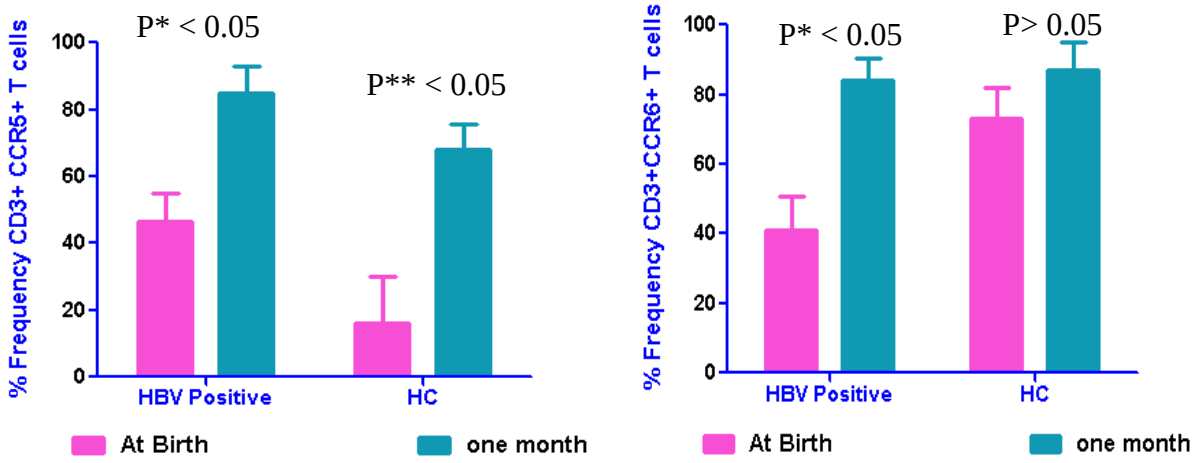


Fig. 5.5.2(G) Increased expression of Chemokine Receptor (CCR1, CCR3) on T cells



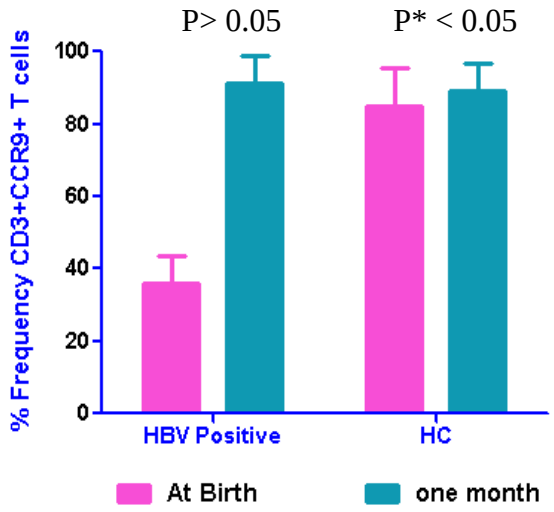


Fig. 5.5.2(H) Increased expression of Chemokine Receptor (CCR5, CCR6, CCR9) on T cells

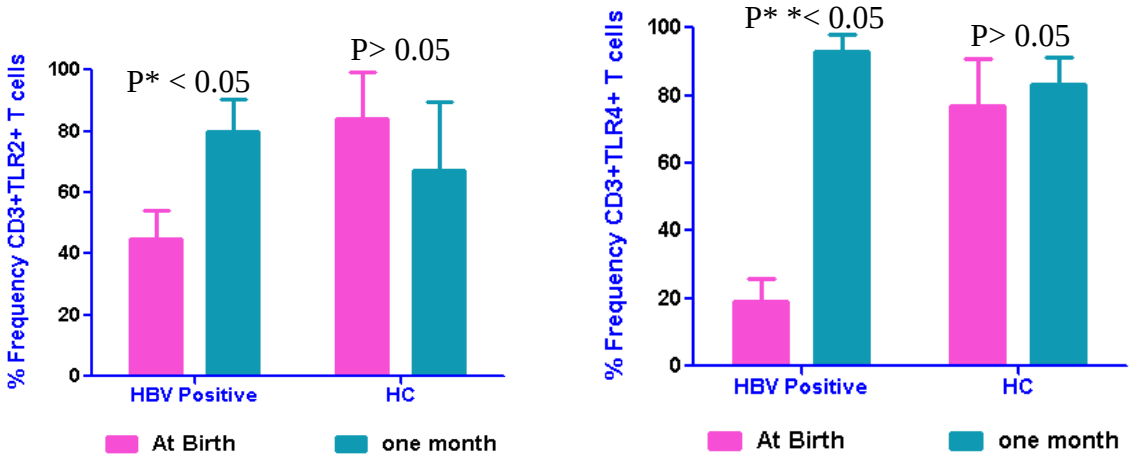


Fig. 5.5.2(I) Increased expression of Toll like Receptor (TLR2 and TLR4) on T cells

Parameter	Baseline %frequency Median	S.E	Day14 %frequency Median	SE	Week 10 % frequency Median	SE	P value Baseline vs. day14	P value Baseline vs. week 10
CD3+CD8+T cells	11.935	11.28	30.77	8.98	17.475	8.15	**P >0.05**	**P >0.05**
CD3+CD4+T cells	44.37	13.56	60.75	10.33	73.76	9.94	**P >0.05**	**P >0.05**
CD8+CD45RA+T cells	0.9219	11.8	87.25	7.26	96.08	15.8	**P* = 0.008**	**P=0.04**
CD8+CD45RO+Tcells	94.6	12.26	12.75	7.38	3.92	15.6	**P* = 0.008**	**P =0.04**
CD4+CCR1+T cells	0.83395	6.25	93.54	2.55	93.88	16.3	**P **= 0.008**	**P>0.05**
CD8+CCR1+T cells	0.98485	10.78	92.24	4.24	83.275	12.98	**P** = 0.01**	**P** = 0.02**
CD4+CCR3+T cells	0.42855	1.67	88.53	13.28	89	16.63	**P **= 0.008**	**P *= 0.01**
CD8+CCR3+T cells	0.9221	3.54	80.95	13.0	78.19	11.97	**P** = 0.008**	**P** = 0.02**
CD4+CCR9+T cells	33.79	16.06	92.025	4.95	77.65	14.36	**P* = 0.01**	**P =0.05**
CD8+CCR9+T cells	39.135	10.29	88.03	5.87	95.27	11.04	**P* =0.01**	**P >0.05**
CD4+TLR2+T cells	18.995	9.8	95.195	8.89	88.33	13.74	**P** = 0.008**	**P* = 0.007**
CD8+TLR2+T cells	69.53	13.88	89.81	10.00	85.415	12.27	**P =0.04**	**P >0.05**
CD4+TLR4+T cells	8.175	5.18	94.33	6.11	74.125	16.2	**P **= 0.001**	**P >0.05**
CD8+TLR4+T cells	49.42	10.23	85.09	11.53	83.09	12.86	**P >0.05**	**P >0.05**
CD4+TLR9+T cells	81.665	8.9	98.495	2.9	75.53	14.7	**P *= 0.05**	**P >0.05**
CD8+TLR9+ T cells	88.835	11.84	98.39	1.23	77.675	5.7	**P*=0.01**	**P>0.05**
CD4+CD25+FOXP3+ T cells	57.14	9.29	56.4	15.6	4.47	10.87	**P >0.05**	**P >0.05**

Table 5.5 Immune profiles of HBV positive neonates at birth before vaccination and at day14 and week 10 after vaccination. (N=8)

5.5.3 Increased Chemokine and Toll like receptor on T cells after vaccination in HBV positive newborns. In HBV positive newborns rapid increase in Chemokine and Toll like receptor expression was observed at 14th day post vaccination in comparison to healthy newborns in which there was gradual age dependent increase.

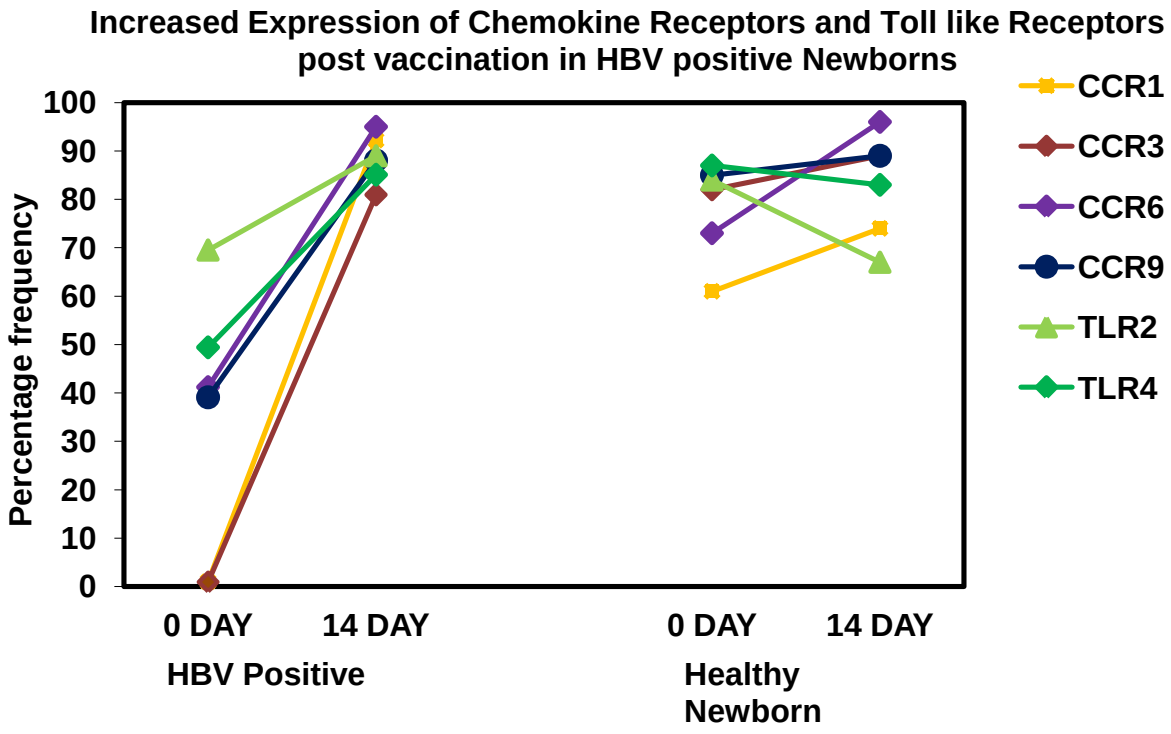


Fig. 5.5.3: Increased Chemokine and Toll like receptor on T cells after vaccination in HBsAg positive newborns. In HBsAg positive newborns rapid increase in naïve T cells, Chemokine and Toll like receptor expression was observed at 14th day post vaccination in comparison to healthy newborns in which there was gradual age dependent increase.

Discussion:

The data presented in this thesis demonstrated that at birth, HBsAg positive newborns born to HBsAg positive mothers have lower proportion of CD4+ and CD8+ T cells, and CD4+T cells were enriched in CD45RO+ memory rather than CD45RA+ naive phenotype compared to HBsAg negative babies. In HBsAg positive newborns significantly higher FOXP3+ regulatory T cells were present compared to HBsAg negative newborns born to HBsAg positive mothers. Studies in mice and humans showed that regulatory T cells suppress the proliferation, cytokine-production (IFN-γ, IL-2), cytolytic activity of naïve and antigen specific CD4+ and CD8+ T cells [Boehmer V. H. et.al, 2005] and functions of antigen presenting cells and B cells through secretion of anti-inflammatory cytokines like IL-10 or TGF-β, direct killing of the target cells or distinct cell-cell contact dependent mechanisms [Pasquetto V, et.al, 2000]. These specialized regulatory T cells could possibly facilitate immune tolerant environment of the newborns preventing the development of mature protective immune response which also support the concept of Medawar hypothesis; "Antigen encountered during fetal life induces a state of acquired immunological tolerance and mammals exposed to foreign homologous tissue cells during fetal life never react immunologically, or react to a limited degree only". Deletion or inactivation of T cells was believed to be the underlying mechanism for such immuntolerance [Gammon G et al. 1986, Zaghouani H et.al, 2009]. Neonatal period has been viewed as a 'window of opportunity' for inducing tolerance to specific antigen. By analogy to T cell tolerance to self Ag, this neonatally-induced unresponsiveness referred to as neonatal tolerance. This probably may leave the newborn as a chronic carrier, at the time of birth.

We have done comparative immunophenotyping of both peripheral and cord blood of HBsAg positive newborns at the time of birth, as there is always a high possibility of contamination of

newborn's cord blood with the mother's blood. Interestingly, no significant differences were observed in the cord blood vs. peripheral blood of newborns at birth which allowed us to analyze the functional properties of the T cells in cord blood, as the peripheral blood sample taken at birth was not sufficient to analyze the functional aspects.

Then phenotypic (CD3ζ) and functional characterization of T cells (IFN-γ production and CD107a cytotoxicity assay) in response to PMA, HBV specific surface and core overlapping pooled peptides was done in HBsAg positive, negative and healthy newborns in their cord blood. We have observed significantly down regulated expression of CD3ζ chain on CD8 T cells which may depict the defects in the mechanism of TCR signaling. Further, down regulation of CD3ζ chain directly correlated with CD8 T cell dysfunction like IFN gamma production and cytotoxicity (measured by CD107a expression). In other settings of chronic inflammation and persistent antigenic stimulation, analogous to CHB, down regulation of CD3ζ has been associated with a CD8 T cell dysfunction. These proximal TCR associated molecules are upstream initiators of signaling cascades, and play a rate-limiting role in efficient T cell signal transduction [Baniyash et.al, 2004] This functional skewing of CD8 T cells could be related to the persistent intrauterine exposure of the viral antigens early in embryonic development leading to immune tolerance to HBV antigens in the HBsAg positive newborns. These observations indicate an ongoing status of established chronic HBV infection and immune tolerant state. These novel observations add a new perspective to our growing understanding of the key mechanisms by which HBV could promote T cell dysfunction related to the loss of CD3ζ chain expression.

Vaccination is an easy and cost effective measure to prevent disease and infection. Additionally, vaccination eliminates the incidences of persistent HBV infection and chronic liver disease and

diminishes the pool of chronic carriers, thus limiting transmission of infection to susceptible contacts [Leuridan E et.al 2011].

Next, to explore the role of HBV vaccine in immune modulation of newborns we have compared pre and post vaccination immune response. We have observed that vaccination considerably augmented the percentage frequencies of CD4+ and CD8+T cells and there was significant increase in CD69+ activated T cell population. However, despite vaccination, no variations were observed in the T regulatory cells population, it was in higher frequencies even after vaccination. Persistent T regulatory cells in HBsAg positive newborns may explain the reason of inability of those newborns to clear the virus and thus becoming immune tolerant.

Chemokines specifically attract and recruit populations of immune effector cells to the sites of injury or infection and their major role is in leukocyte migration and dependent processes such as immune surveillance and innate and adaptive immune responses [Borish, L.C. et.al, 2003, Strieter RM, et.al. 1996]. Data presented in this study indicates that at birth, HBsAg positive newborns have lower proportion of chemokine receptors expressing CD4+T cells, specifically CCR1, CCR3 and CCR9 and were significantly down regulated compared HBsAg negative and healthy newborns. Depressed Chemokine receptor expression suggests us to conclude the inability of T lymphocytes recruitment resulting in defective adaptive immune responses in the newborns with vertically transmitted HBV. There is a significant up-regulation of expression of Chemokine receptors CCR1, CCR3, CCR9 was observed on T cells after HBV vaccination. These findings show a new vista indicating the beneficial role of vaccination to augment the adaptive immunity through increased expression of Chemokine Receptors on T cells.

We have determined the expression of Toll like Receptors on CD8 T cells at birth pre and post vaccination. At birth, in HBsAg positive newborns TLR2, TLR4 and TLR9 were significantly

down regulated on CD8 T cells as compared to healthy newborns. TLRs play a crucial role in early host defense by recognizing so-called pathogen-associated molecular patterns that are essential for the survival of the microorganism but are not present in eukaryotes. They are important in host defense against microbial infection by regulating both innate and acquired immunity [Akira S, et.al 2004, Kaisho T, et.al 2006]. Studies demonstrated the role of TLR3, TLR4, TLR5, TLR7, and TLR9 in complete inhibition of HBV replication in a HBV transgenic mice model [Isogawa et. al. 2005]. TLR4 plays an anti-HBV role in acute HBV expression through induction of i-NOS expression and specific anti-HBV immune responses. TLR4 plays a role in viral infections as in Respiratory syncytial virus (RSV) infection persists longer in the lung of TLR4-deficient mice than normal mice, and RSV fusion protein can activate the human monocytes through TLR4. Post vaccination TLR2, TLR4 and TLR9 were up regulated on T cells thus depicting the role of vaccine in partial improvement of adaptive immune responses.

Our results, thus strongly favor vaccination that is helpful in partial improvement of T cell mediated adaptive immune responses in newborns. In summary, HBV vaccination partially restores depressed adaptive immunity against HBV in newborn through increased expression of CCRs and TLRs whereas sustained expression of T-regulatory cells may play a significant role in the development of chronic HBV infection in these newborns. HBV vaccination is able to only partially restore the host immune response against the infection.

B cell response in HBV infected Newborns

The main cellular components of response of adaptive immune defence against virus infection include T and B lymphocytes [Bertoletti & Gehring 2006]. During Hepatitis B virus infection the adaptive immune response is thought to be responsible for viral clearance and disease pathogenesis. It is generally acknowledged that the humoral antibody response contributes to the clearance of circulating virus particles and the prevention of viral spread within the host while the cellular immune response eliminates infected cells.

A strong multi-specific CD8 T cell response, with adequate CD4 T cell help and co-ordinated humoral immunity, provides a solid adaptive immune defence against virus infection [Bertoletti & Gehring 2006]. The main cellular components of this response include B and T lymphocytes. During Hepatitis B virus infection the adaptive immune response is thought to be responsible for viral clearance and disease pathogenesis. It is generally acknowledged that the humoral antibody response contributes to the clearance of circulating virus particles and the prevention of viral spread within the host while the cellular immune response eliminates infected cells.

B cells represent an important arm of adaptive immune response which play central roles in the establishment and maintenance of protective immunity against pathogens, including the generation of protective antibodies, antigen presentation, and more recently, appreciated regulatory functions [Chung JB et.al 2003]. Self-limiting viral infections are characterized by migration of naïve B cells to lymph nodes where they encounter T cells, become activated and are selected for high-affinity antigen-binding in germinal centers, from which they are eventually released as long-lived resting memory B cells or plasma cells [LeBien T W. et.al 2008]. Whereas persistent viral infections are accompanied by increased number of activated and exhausted B

cells, increased levels of short lived plasma B cells or immature transitional B cells associated with CD4 T cell lymphopenia or decreased memory B cell response as elegantly shown in HCV and HIV infections [Moir S et.al, 2009]. There are studies that demonstrate the role of overwhelming B cell response in pathogenesis of HBV associated acute Liver Failure with massive intrahepatic production of IgM and IgG by plasma cells infiltrating the hepatic lobules centered in the liver [Farci P et.al 2010].

Peripheral B cells can be broadly characterized into 3 main groups; transitional, mature and memory dependent on expression of surface markers CD19, CD24 and CD38 [Carsetti R et.al 2004]. To identify human transitional B cells, two developmentally regulated markers, CD24 and CD38, in combination with the B-lineage marker CD19 are used. In the peripheral blood, all cells of the B lineage (CD19pos) co express CD24 and CD38, and, conversely, all non-B cells (CD19neg) lack CD24. Three populations of B cells can be discriminated based on the relative distribution of CD24 and CD38. The CD24brightCD38neg population included 60% of all B cells in this donor, 58% of the B cells were CD24dullCD38pos, and only 2% expressed high levels of both CD24 and CD38 (CD24brightCD38bright. Essentially all CD24brightCD38neg cells are memory B cells, and mature B cells correspond to the CD24dullCD38pos population. CD24brightCD38bright cells lack CD27.

In the first two objectives, we have discussed about the T cell response in the HBV infected and non-infected newborns at birth pre vaccination and post vaccination at different time points. We next aimed to explore the potential immune mechanisms of maternal fetal transmission of HBV associated with the underlying B cell defects in the newborns vertically infected from their mothers. Thus, we have characterized the B cell profile in HBV infected and non-infected

newborns born to HBV positive mother and further we have investigated the changes in B cell profile and various subset distributions at 12 months post vaccination.

5.6 Dominance of regulatory transitional B cells and lower memory B cells in HBsAg positive compared to negative newborns.

Peripheral B cells can be broadly characterized into 3 main groups; transitional, mature and memory dependent on expression of surface markers CD19, CD24 and CD38. B cells were identified based on CD19 and classified by multiparameter flow cytometry based on the expression of defined surface markers as follows: transitional (CD19+CD38hiCD24hi), memory (CD19+CD24hiCD27+) and plasma (CD19+CD38+) B cells [Figure 7.1(A-C)]. At Birth before vaccination, HBsAg positive newborns showed significantly higher proportion of transitional B cells and lower memory B cells (CD19+CD24hiCD27+) compared to HBsAg negative newborns (total 7.4% vs. 9.6% P=NS, transitional 2.7% vs. 0.06%, P***=0.0002; memory 4.9% vs. 7.4% P=NS, P**=0.005, plasma B cells 2.2% vs. 1.8% P=NS). [Figure 5.6(D)].

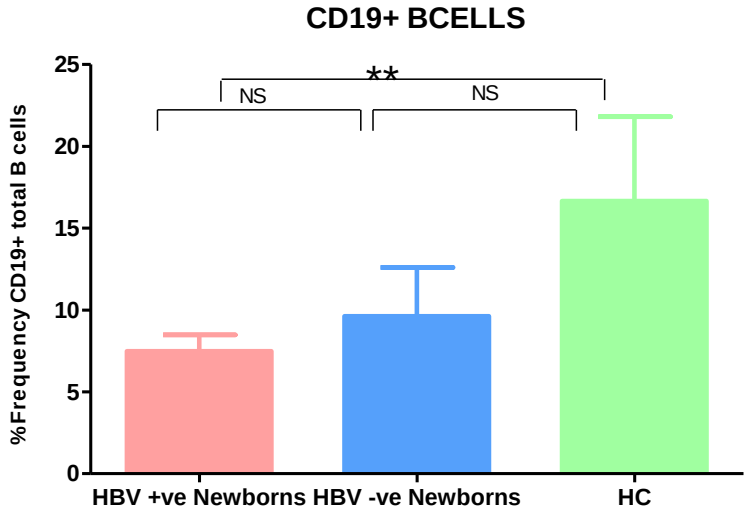


Figure 5.6(A)

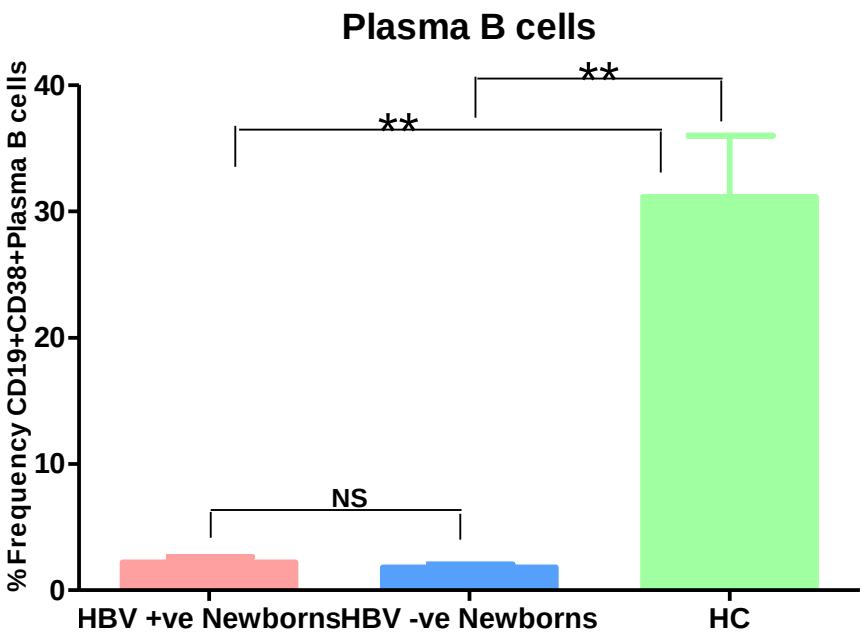


Figure 5.6(B)

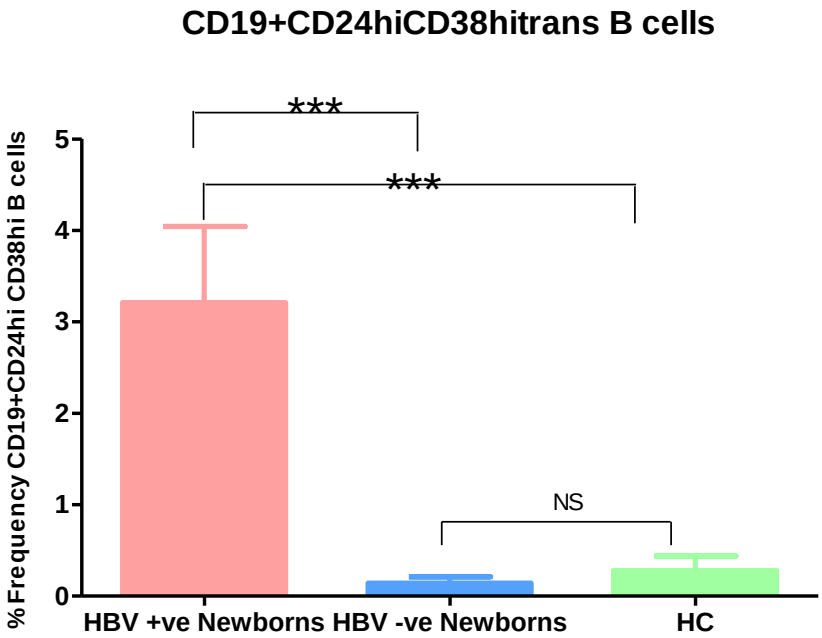


Figure 5.6(C)

Figure 5.6(A-C): Dominance of immunosuppressive transitional B cells in HBV positive compared to negative newborns : At Birth before vaccination HBV positive newborns showed significantly higher levels of regulatory transitional B cells (CD19+CD24hiCD38hi) and lower memory B cells (CD19+CD24hi) compared to HBV negative newborns (transitional 2.7% vs. 0.06%, P***=0.0002; memory 4.9% vs. 7.4%, P**=0.005).

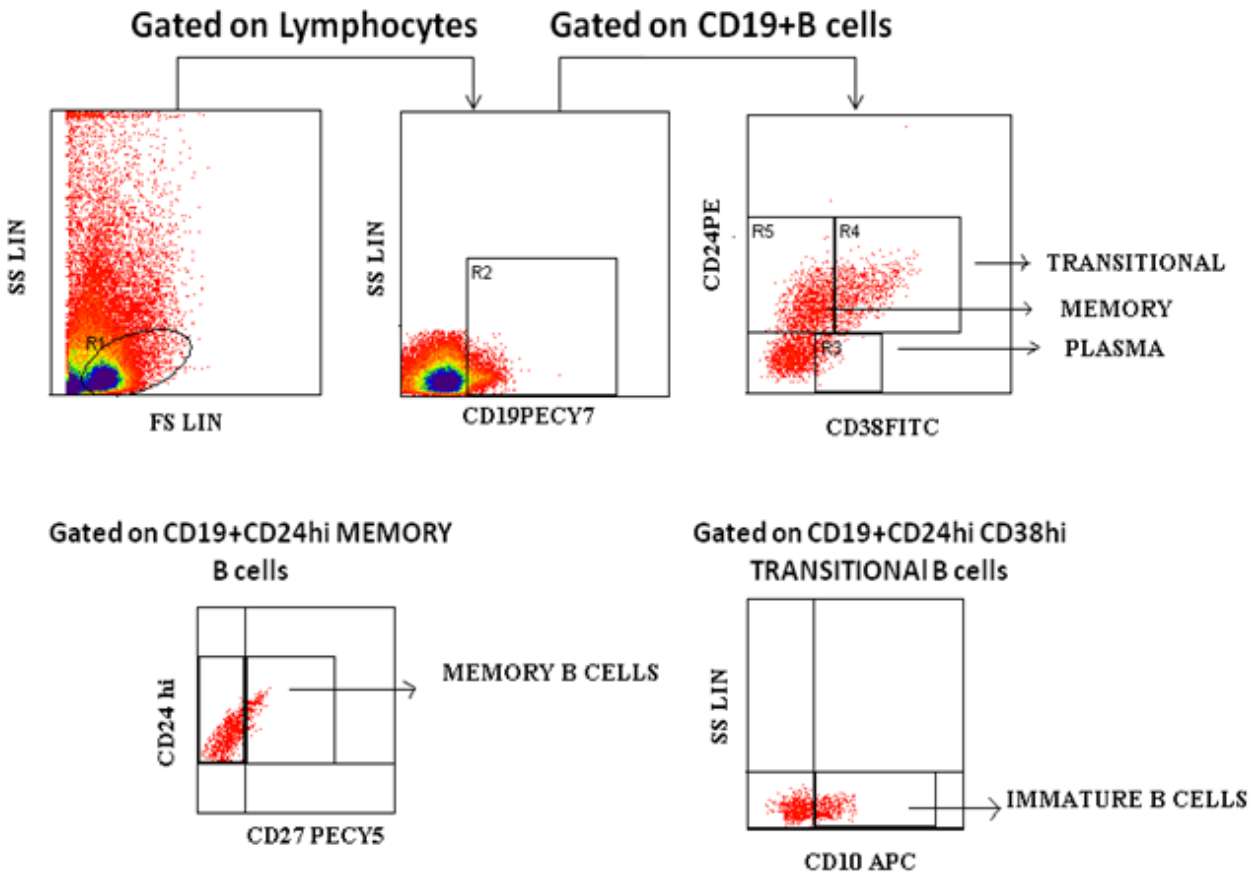


Figure 5.6(D)

Figure 5.6(D): Gating strategy used for different B cell subset frequency determination: Flow cytometry was performed for identification and determination of frequencies of different B cell sub-populations which were identified based on CD19 expression and classified by multiparameter flow cytometry based on the expression of defined surface markers as follows: Immature (CD38hiCD24hiCD10+), Transitional (CD19+CD38hiCD24hi), Memory (CD19+CD4hi CD27+). Plasma B cells (CD19+CD38+).

5.7 Post-vaccination declination of Transitional B cell population and increase in memory B cells in HBV positive newborns

We, then next evaluated the influence of HBV vaccination on these newborns in correlation with development of protective B cell response. Interestingly, we found that at 12 months post vaccination, a decline in transitional B cell population and an increase in memory B cells were observed in HBV positive newborns compared to at birth before vaccination. After vaccination, no significant differences were observed among HBV positive and negative newborns; Gr. I at birth vs. 12 months: (Transitional 3.2% vs. 1.09% P*=0.04, memory 5.1% vs. 9.73% P*=0.03). Gr.I vs. Gr. II: (transitional 1.09%vs. 2.13% P=NS, memory 9.73%vs. 6.18% P=NS) Fig. 5.7 (D-F)].

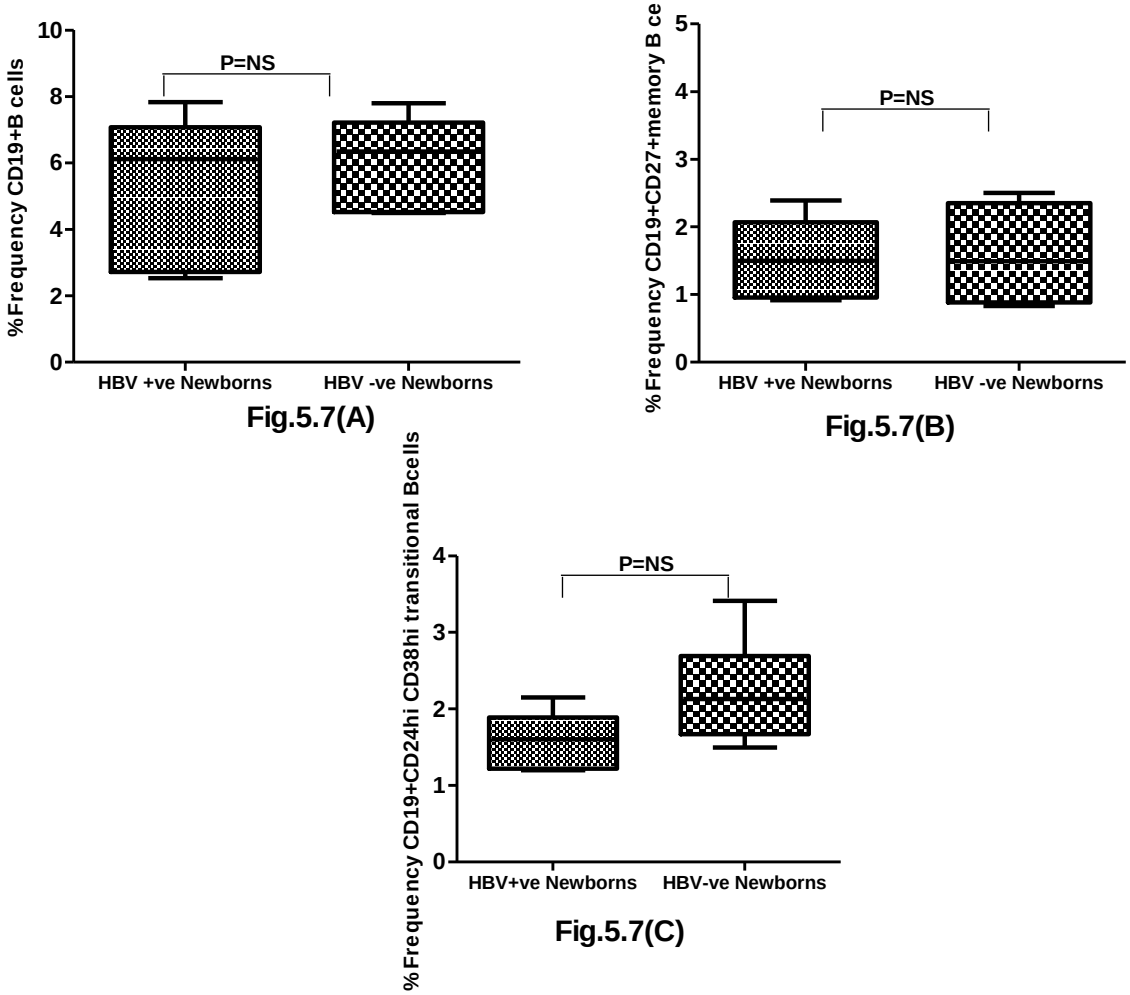


Fig.5.7(A)

Fig.5.7(B)

Fig.5.7(C)

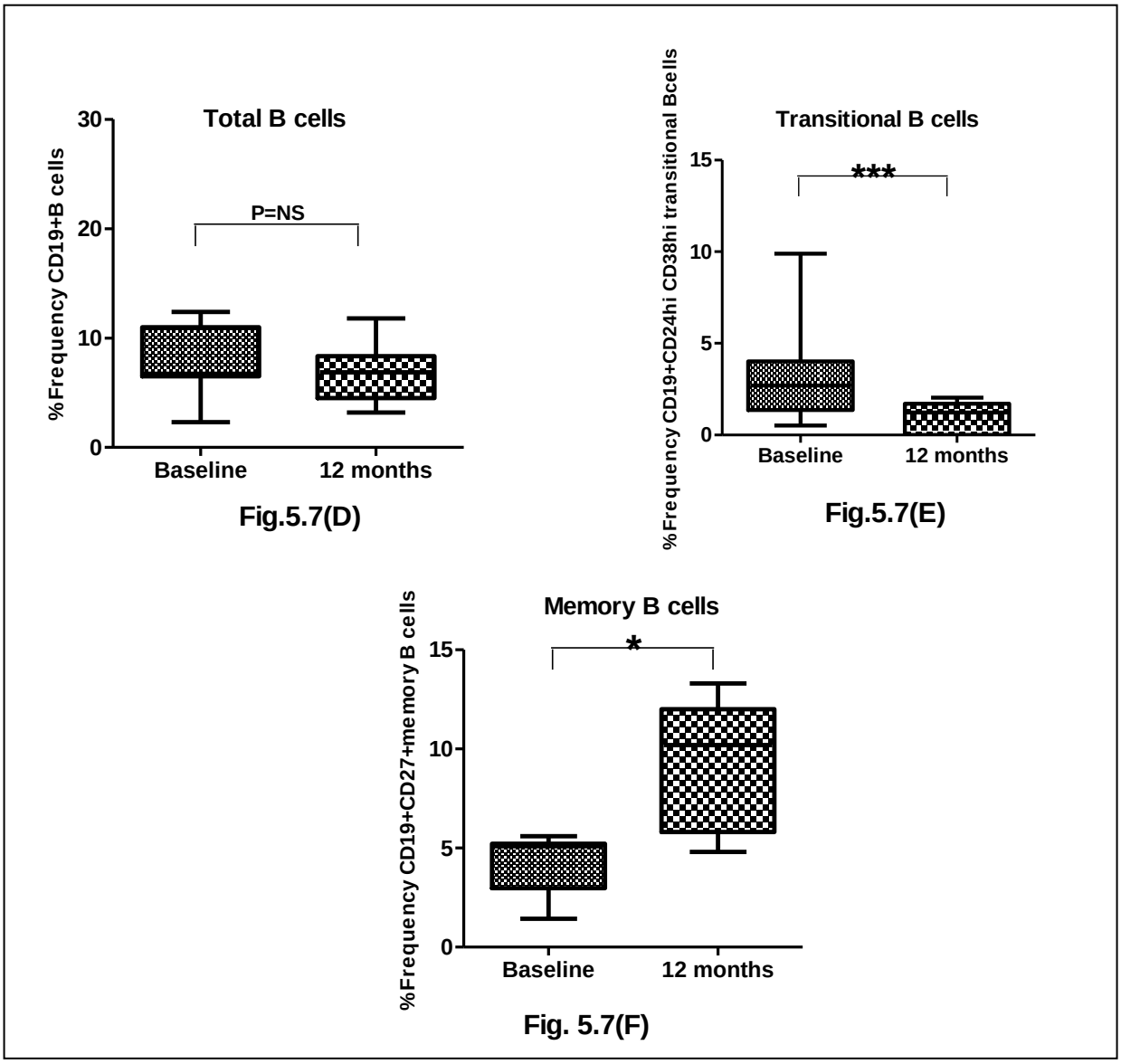


Figure 5.7 (A-F) Post-vaccination declination of Transitional B cell population and increase in memory B cells in HBV positive newborns: At 12 months post vaccination, a decline in transitional B cell population and an increase in memory B cells were observed in HBV infected newborns while no significant differences were observed when compared with the non-infected newborns. Representative bar graphs showing the differences in the frequencies of B cell subsets. Gr.I vs. Gr.II: (transitional 1.09%vs. 2.13%, memory 9.73%vs. 6.18% P=NS); Gr. I at birth vs. 12 months :(transitional 3.2% vs. 1.09% P*=0.04, memory 5.1% vs. 9.73% P*=0.03).

5.8 Post vaccination enhanced expression of activation marker CD69 and Chemokine receptor CCR5 on memory B cells in HBV positive newborns

For further delineating the phenotype of the expanded population, we have determined the differences in the expression of activation marker CD69, Chemokine receptor CCR5, PD1 which is an inhibitory molecule, and TALL-1 (TNF- and ApoL-related leukocyte expressed ligand-1) which regulates B cell survival and expansion by flow cytometry on the different B cell subpopulation, total (CD19+) B cells, naïve (CD19+CD27-) B cells and memory (CD19+CD27+) B cells. HBV infected newborns displayed increased expression of activation marker CD69 and Chemokine receptor CCR5 on memory (CD19+CD27+) B cells while there were no differences observed in the total and naïve B cell compartment at 12 months post vaccination compared to at birth before vaccination. We then evaluated the phenotypic expression of PD1 and TALL-1 on the various B cell subpopulations and observed that both the groups expressed comparable levels of TALL-1 and PD-1 on total, naïve and memory B cells. (Gr. I vs. Gr. II: CD69+: 5.7% vs. 0.67% P*= 0.02 P*= 0.03, CCR5+: 6.3% vs. 0.78% P*= 0.002, TALL-1+:32% vs. 29% P=NS, PD1+ 0.97% vs. 0.94% P=NS) [Fig.5.8 (A-D)]. Gating strategy used to analyze the expression of indicated molecules on different B cell subpopulation is described in representative FACS dot plot analyzed by Summit v4.3Software [Fig.5.8 (E)].

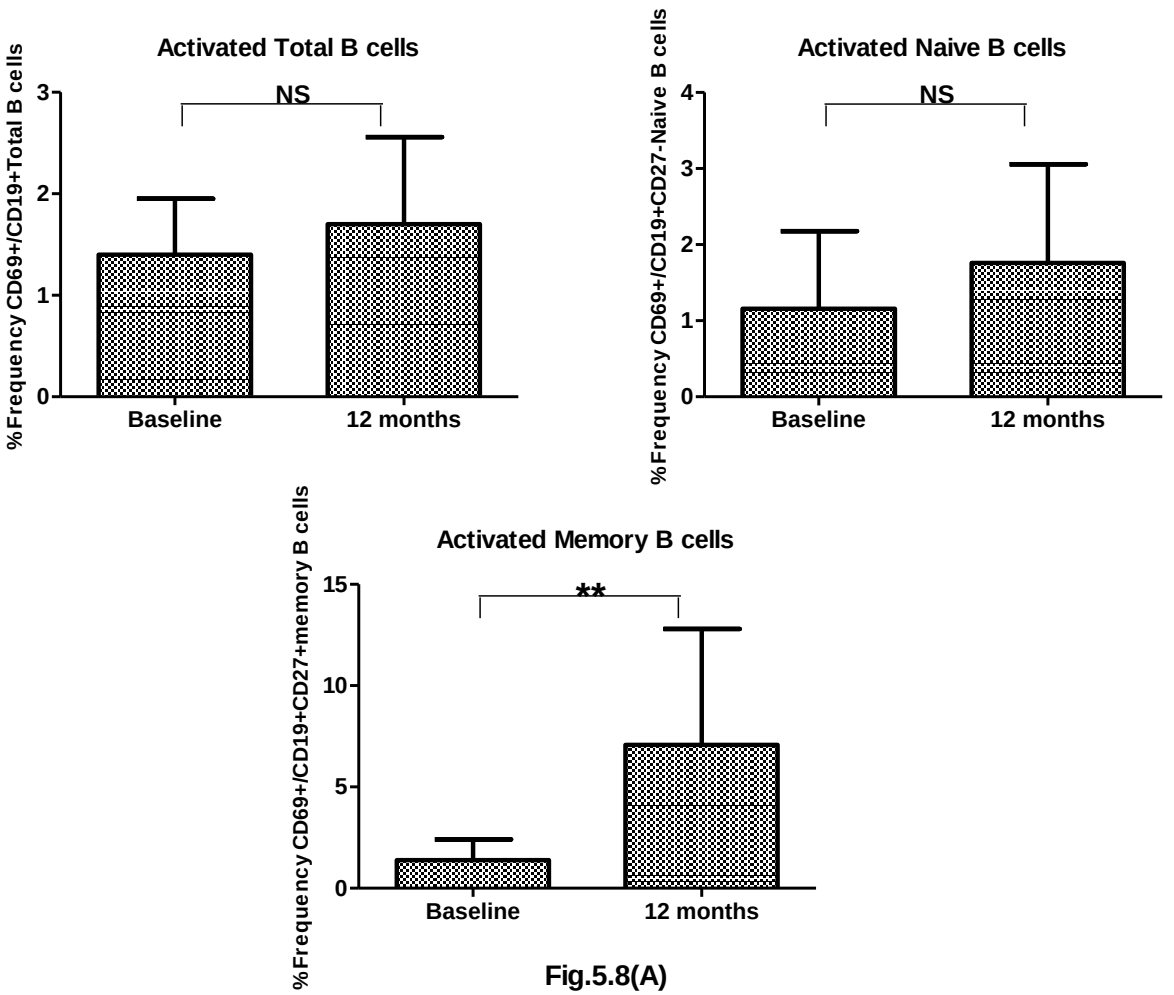

Activated Total B cells
% Frequency CD69+/CD19+Total B cells
NS
3
2
1
0
Baseline
12 months
Activated Naive B cells
% Frequency CD69+/CD19+CD27-Naive B cells
NS
4
3
2
1
0
Baseline
12 months
Activated Memory B cells
% Frequency CD69+/CD19+CD27+memory B cells
**
15
10
5
0
Baseline
12 months

Fig.5.8(A)

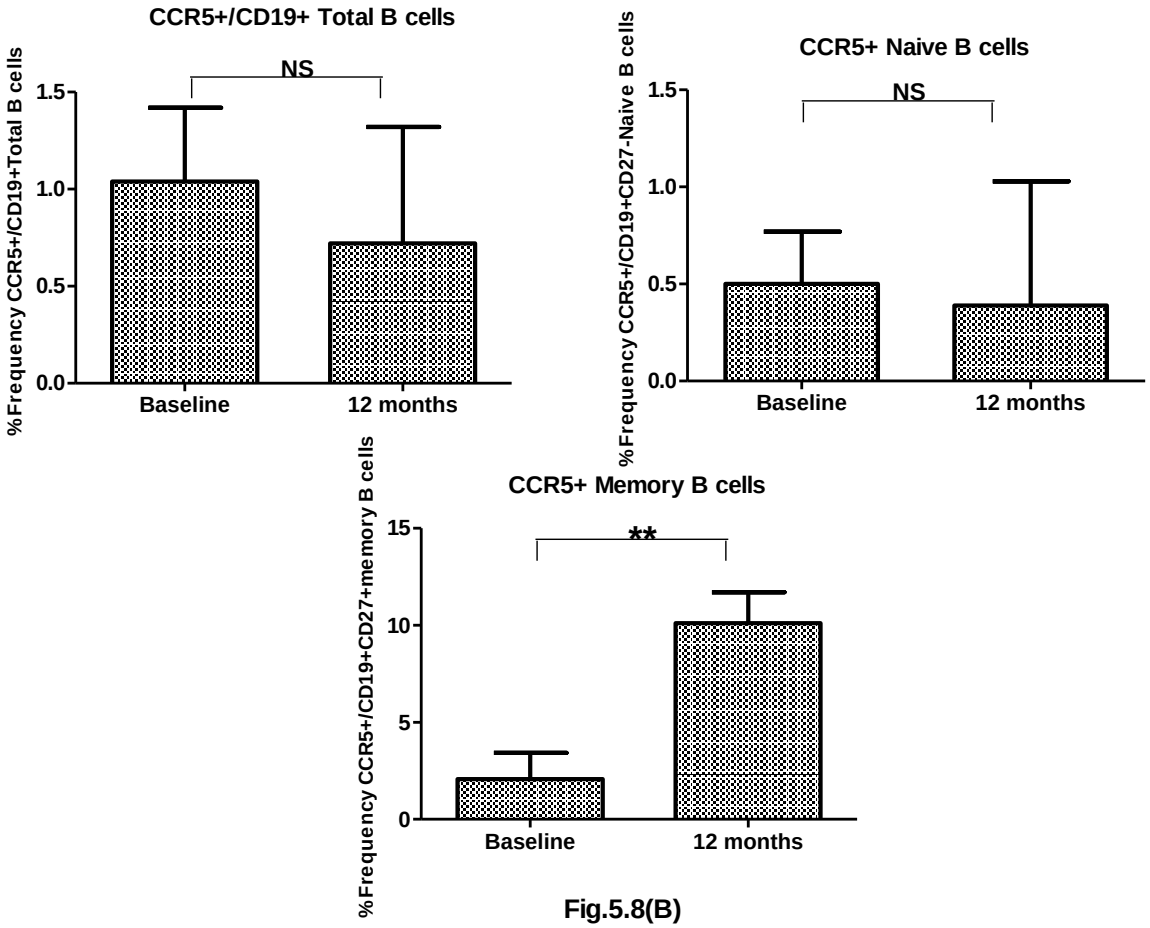
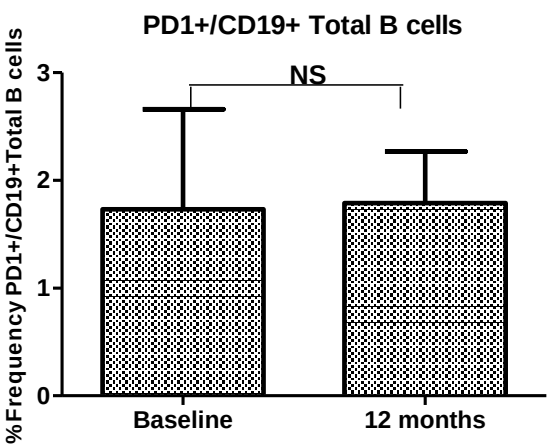
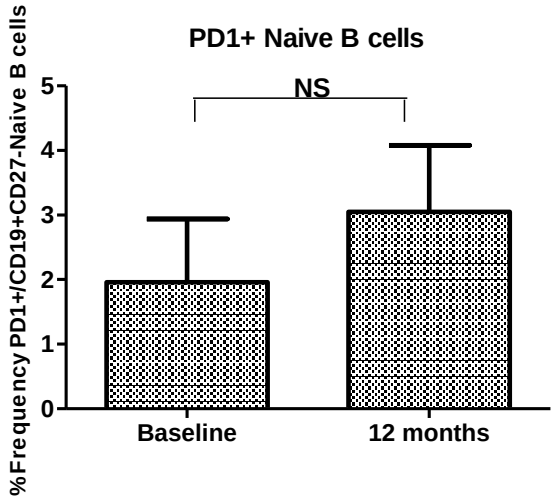

CCR5+/CD19+ Total B cells
%Frequency CCR5+/CD19+Total B cells
NS
1.5
1.0
0.5
0.0
Baseline
12 months
CCR5+ Naive B cells
%Frequency CCR5+/CD19+CD27-Naive B cells
NS
1.5
1.0
0.5
0.0
Baseline
12 months
CCR5+ Memory B cells
%Frequency CCR5+/CD19+CD27+memory B cells
**
15
10
5
0
Baseline
12 months

Fig.5.8(B)


PD1+/CD19+ Total B cells
NS
% Frequency PD1+/CD19+Total B cells
3
2
1
0
Baseline
12 months


PD1+ Naive B cells
NS
% Frequency PD1+/CD19+CD27-Naive B cells
5
4
3
2
1
0
Baseline
12 months

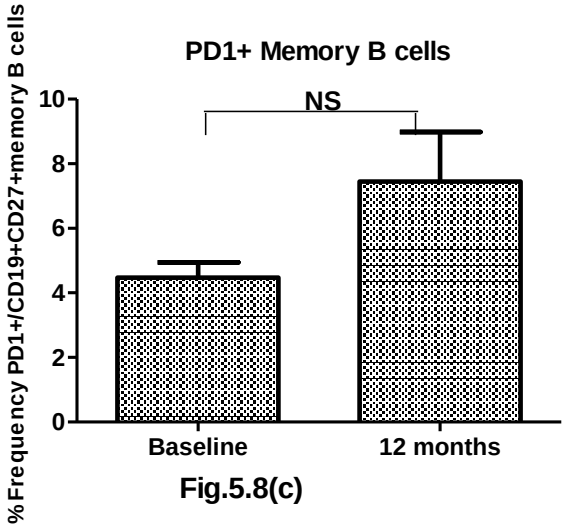

PD1+ Memory B cells
NS
% Frequency PD1+/CD19+CD27+memory B cells
10
8
6
4
2
0
Baseline
12 months

Fig.5.8(c)

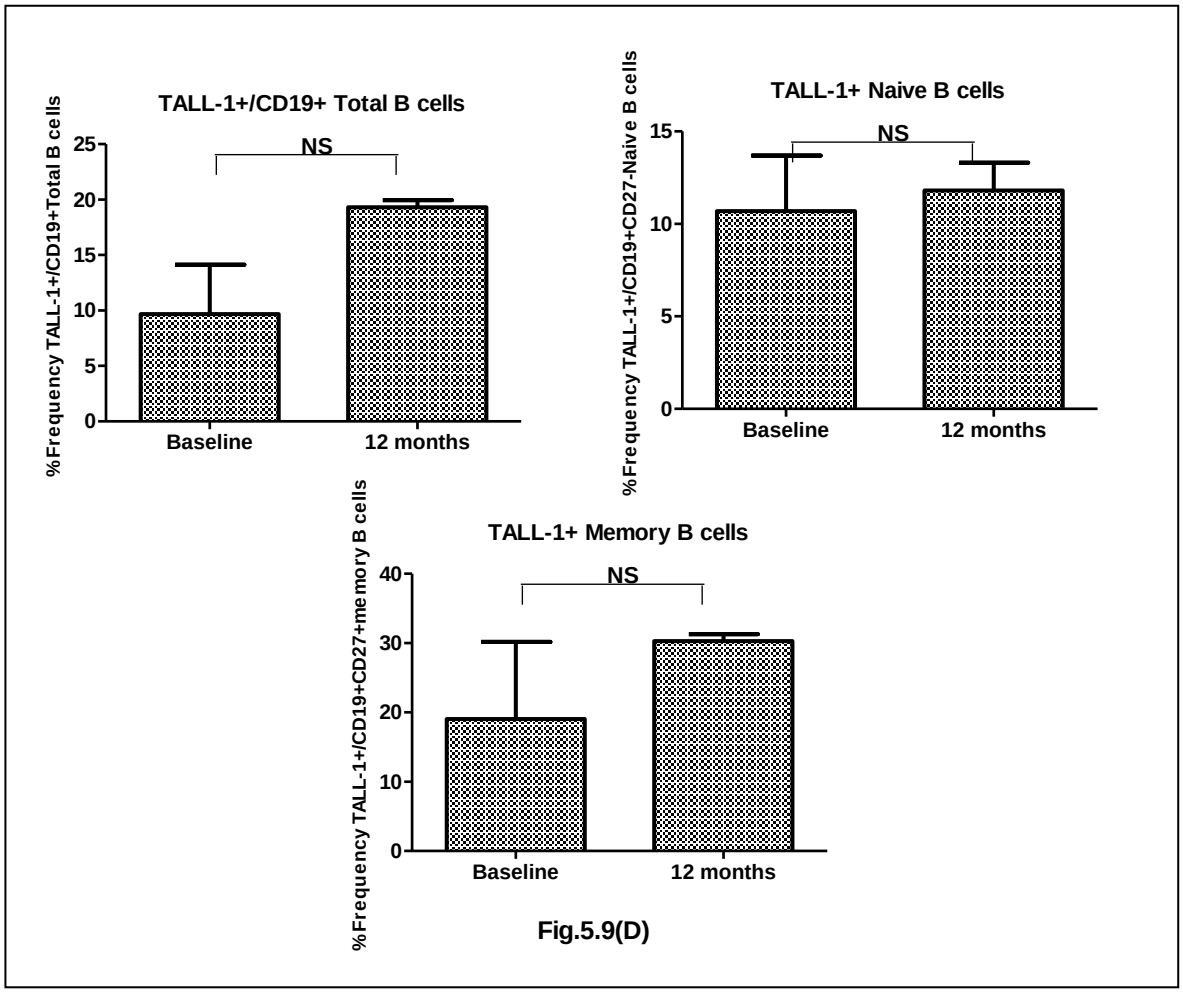


Fig.5.9(D)

Figure 5.9 (A-D) Post vaccination enhanced expression of activation marker CD69 and Chemokine receptor CCR5 on memory B cells in HBV positive newborns B lymphocytes from HBV positive newborns at birth pre-vaccination and at 12 months post vaccination were analyzed ex vivo for surface expression of activation marker CD69, Chemokine receptor CCR5(homing receptor) , inhibitory molecule PD-1 and TALL-1 (TNF- and ApoL-related leukocyte expressed ligand-1) which regulates B cell survival and expansion by flow cytometry. Data is shown as the percentage of total (CD19+), memory (CD19+/CD27+), or naïve (CD19+/CD27-) B cells expressing the indicated molecules.

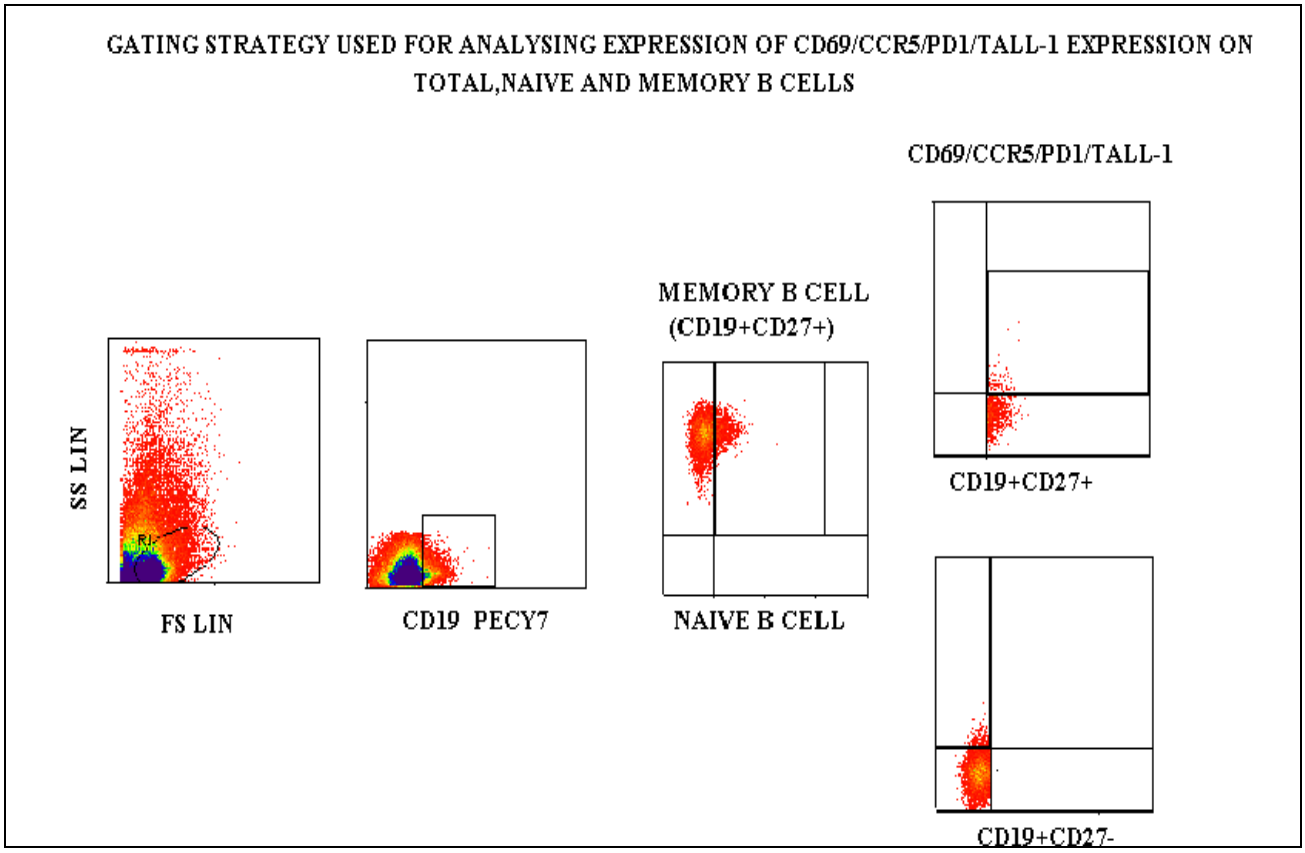


Fig.5.9 (E)

Fig.5.9(E):Gating strategy used to analyze the expression of different CD69/CCR5/PD1/TALL-1 on different B cell subpopulation Flow cytometry was performed for evaluating the expression of activation marker CD69, Chemokine receptor CCR5, PD1 which is an inhibitory molecule, and TALL-1 (TNF- and ApoL-related leukocyte expressed ligand-1) which regulates B cell survival and expansion by flow cytometry on the different B cell subpopulation [total (CD19+) B cells, naïve (CD19+CD27-) and memory (CD19+CD27+) B cells]. Samples were acquired on Beckman Coulter CYAN-ADP Flow cytometer and analyzed by Summit v4.3Software described in representative FACS dot plot.

DISCUSSION:

From the past many years intense research efforts have been dedicated to elucidating the pathogenic mechanisms of HBV-associated disease progression. Several investigators have described the host immune defects underlying immune response to HBV infection in the adults, but there are limited studies done on immune profiles of newborns during vertical transmission from their HBV positive mothers. In the previous chapter, we have determined the T cell defects in the HBV positive newborns and provided deep insights into the mechanisms of chronicity in the newborns related to diminished expression of T cell receptor zeta chain accompanied with reduced IFN gamma production and cytotoxicity capacity of CD8 T cells along with predominance of T regulatory cells in HBV positive compared to HBV negative newborns.

In addition to the progressive depletion and dysfunction of CD8+ T cells, Chronic HBV infection also leads to extensive defects in the humoral arm of the immune system. The humoral immune response is vital for long-term clearance of HBV and protection from infection which commonly seems to be compromised in infants [Chang J.J. et.al, 2007]. In patients who recover from acute HBV infection, activated T-helper cell type 2 (Th2) CD4+ T-cells induce B-cell production of HBV specific antibodies that are important in providing protective immunity against subsequent HBV infections and the basis of protection in vaccinated individuals [F.V. Chisari et.al 1995,]. In infants, B cell mediated antibody responses are typically of shorter duration, have a delayed onset, differ in the distribution of immunoglobulin G (IgG) isotypes (lower titers of IgG2) and are of lower affinity than are adult responses [Siegrist C.A. et.al, 2009].

We have observed significantly higher levels of transitional B cells (CD19+CD24hiCD38hi) in the HBV infected newborns compared to the non infected newborns. Transitional B cells are functionally immature subset has been shown to suppress immune responses in situations of

chronic inflammation including chronic HBV infection [Chung J.B. et.al 2003]. Many studies have documented the presence of large numbers of transitional B cells (with high expression of CD5, CD10, CD24 and CD38), during early infancy [Prabhu Dass M et.al, 2011]. Our study reports further increase in the immature or transitional B cell population in the HBV positive newborns which may explain the possible mechanism of acquisition or persistence of HBV in them compared to negative newborns.

For antibody responses, memory is encoded, in part, in long lived memory B cells. Memory B Cells after activation can proliferate and secrete high levels of immunoglobulins during antigenic challenge. Immunological memory, the ability to respond more rapidly and robustly to re-exposure to an antigen, is a hallmark of adaptive immunity [Fecteau J.F.et.al, 2009]. We have observed significantly lower memory B cell response in HBV positive newborns compared to negative newborns which could be directly correlated with the inability of positive babies to clear the virus and get the infection.

We, then next evaluated the influence of HBV vaccination on these newborns in correlation with development of protective B cell response. Here we have seen marked declination in the immunosuppressive transitional B cell population after 12 months of vaccination which was predominantly present at birth in the positive newborns and increase in the memory B cells which was lower at birth. Declination of functional immature or limited transitional B cell population and increase in the memory B cells indirectly reflect the beneficial role of HBV vaccine. Moreover, after complete vaccination we have observed expansion of CD69+ activated memory, CCR5+ memory B cells in the infected newborns implicating the development of protective B cell response against the virus in contrast to the comparable levels of TALL-1 and PD-1 on memory B cells.

Further, we need to determine the functional implication of these altered B cell response in the newborns.

Thus our data indicates that there are marked distinctions in the B cell biology (B cell development and responses) among both the groups associated with the predominance of immature transitional B cells and lower memory B cell response in these newborns which may partially explain the reason of HBV persistence in HBV positive newborns. Additionally, this study supports vaccine in augmenting the memory B cell response and decreasing the functionally limited immunosuppressive transitional B cell population.