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Cell-Mediated Immune Responses in Neonates

1. INTRODUCTION

Cell-mediated immune (CMI) responses are mediated by T cells. T-cell subsets, such as Th1 and Th2, exhibit various functions. CD4 Th1 cells are the effectors of delayed-type hypersensitivity reactions, producing cytokines such as IFN-γ, which activates macrophage to kill obligatory intracellular pathogens, and interleukin (IL)-2, which promotes the growth of CD8 T cells. Th1 cells also promote class switching of IgM to IgG2. Th2 cells secrete cytokines to promote the B-cell activation and class switching of IgM to IgG1 and IgE. The CD8 cytotoxic T cells (CTLs) lyse cells infected with microbes (viruses, intracellular bacteria, and parasites) as well as cells expressing tumor-associated antigens. Therefore, CMI responses play an important role in immune defense reactions.

For a long time, it was generally believed that newborns exhibit an increased susceptibility to infection because of immaturity of neonatal lymphocytes, deficiency of antigen-presenting cell (APC) function, dominance of Th2 response, and the induction of tolerance. The concept of the immaturity of newborn T cells was supported by the observation that neonatal lymphocytes proliferate less than adult lymphocytes following stimulation of T-cell receptor with anti-CD3 antibodies *(151)*. The concept of high susceptibility to tolerance of neonatal T cells originated from the experiment by Billingham et al. *(607)* demonstrating that injection of newborn mice with allogenic cells prevents rejection of an allograft.

Fervent development of cellular, clinical, and molecular immunology during the past 50 yr has provided new findings leading to the revision of the concept that neonatal T cells can not mount cellular immune responses. These findings show that CMI responses in neonates could be induced in certain conditions by varying the dose of the immunogen,employing new antigen delivery systems, and using cytokines or costimulatory factors. Clinical studies also contributed by demonstrating that neonates and infants can mount efficient CMI responses following natural infections with bacteria or viruses and vaccinations with live-attenuated vaccines.

From: *Contemporary Immunology: Neonatal Immunity*
By: Constantin Bona © Humana Press Inc., Totowa, NJ

2. NEONATAL TOLERANCE TO ALLOANTIGENS IS NOT AN INTRINSIC PROPERTY OF NEWBORN LYMPHOCYTES

The CMI response to alloantigens is responsible for the rejection of allografts. CD8 T cells mediate graft rejection when the graft differs from the recipient only in major histocompatibility complex (MHC) class I alloantigen. Although CD4 T cells do not play a direct role in allograft destruction, they augment the CD8 T-cell activity by providing cytokines, such IL-2, that are necessary for the expansion of CD8 T cells. The CD8 T cells are expanded during graft rejection subsequent to recognition of peptides derived from processing of MHC class I molecules. In contrast, CD4 T cells may mediate the rejection of allografts bearing MHC class II antigens different from those of the recipient.

In the mouse, the precursors of alloantigen-specific CTLs were detected in the thymus of newborns and in the spleen of mice age 3–9 d *(1052,1053)*. It was believed that the precursors of alloantigen-specific CTLs exhibit an intrinsic tolerogenic property at birth, because injections of a high number of bone marrow allogenic cells prevented graft rejection of skin and consequently prevented a CTL response *(607)*. Ridge et al. *(1054)* analyzed CTL induction in female newborn B6 mice injected with male cells and tested the CTL response against male H-Y antigen. In this system, the in vitro H-Y-specific CTL response is completely dependent on in vivo immunization with alloantigen. Like in Billingam's experiment, tolerance was induced after injection of a high number of male cells (i.e., five male spleen cells to one female T cell). However, when females were injected at birth, not with male spleen cells but with male dendritic cells, a CTL response was observed. The CTL activity was detected 8 wk after injection, indicating that dendritic cells from adult males efficiently primed the precursors of H-Y CTLs at birth.

This finding demonstrates that mice receiving dendritic cells from an adult donor were resistant to tolerance induction and that the CTLs were primed to the same extent as adult female controls that were injected with male cells. In addition, this experiment showed that neonatal alloreactive T cells do not have an intrinsic property for tolerization but rather the immaturity of APCs controls induction of neonatal tolerance. It was shown that human cord blood dendritic cells have a poor capacity to stimulate naïve alloantigen-specific T cells compared to adult dendritic cells *(1055)*. The tolerance induced with bone marrow cells might be related to the high number of immature dendritic cells, which are unable to deliver costimulatory signals to alloantigen-specific naïve T cells in newborns. The requirement of costimulatory signals for neonatal naïve T cells to reach adult levels of function is supported by the in vivo requirement of multiple vaccine doses for infants *(1056)* and by the finding that CD40 binding prevents neonatal tolerance *(946)*.

3. NEONATAL T CELLS ARE ABLE TO MOUNT A TH1 RESPONSE IN CERTAIN CONDITIONS

Studies of neonatal T-cell function showed that the response to foreign antigen is skewed toward a Th2 dominant response in both mice and humans. In mice, immunization as early as the first week of life with vaccines such as tetanus toxoid, live-attenuated measles virus, or BCG-induced higher IgG2a antibody response, significantly higher IL-5 production, and lower IFN-γ synthesis by antigen-specific T cells. This pattern of response was maintained in adults, as assessed by measuring the response elicited by boosting with the corresponding vaccines *(645)*. However, recent studies have challenged this concept, demonstrating that under certain experimental conditions or during infection with certain microbes, Th1 responses might be induced in newborns, reaching into adulthood.

Several studies performed on newborn mice documented the ability to induce both Th1 and Th2 immunity. Injection of mice after birth with soluble hen egg lysozyme (HEL) protein induced tolerance; however, intraperitoneal immunization with HEL in incomplete adjuvant expanded cells that produce IFN-γ and IL-5 and favored the synthesis of both IgG1 and IgG2a anti-HEL antibodies *(945)*. The results of this study clearly showed that neonatal CD4 T cells are immunocompetent and that immunization with antigen and adjuvants elicits both Th1 and Th2 responses. A mixed Th1/Th2 response was also observed in mice immunized with a *Helicobacter pylori* extract in either complete or incomplete Freund's adjuvant. Although gastric diseases are typically manifested in adults, infection with *H. pylori*, which causes chronic gastric diseases, occurs in children under age 5 yr *(1057,1058)*. Newborn mice immunized with *H. pylori* lysates in adjuvant exhibit a protective immunity, as assessed by a decreased bacterial load in the stomach, a lower gastritis score, and a higher frequency of cells producing IFN-γ, IL-2, and IL-4 in recall assay *(1059)*.

The induction of neonatal Th1 responses by viruses depends on the replication capacity of virus in the host cells. This was clearly demonstrated in a study of protective immunity induced in neonatal mice by immunization with a single replicative cycle of herpes simplex virus (HSV) called disabled infectious single-cycle HSV variant (DISC). This variant lacks the gene that encodes glycoprotein H, which is essential for infection. Mice immunized 24 h after birth with DISC-HSV-1, but not with UV-inactivated HSV-1 virus, were protected from lethal HSV-1 infection, and the protection could be transferred to naïve recipients with both CD4 and CD8 T cells. The protective effect of CD4 T cells was related to Th1-mediated immune responses by IFN-dependent and -independent mechanisms. The protective function of transferred CD8 T cells might be caused directly by cytolytic activity or indirectly by secretion of IFN-γ cytokine *(1060)*. Similar results were obtained by immunizing newborn mice

with a noninfectious strain of Sendai virus TR-5, a cleavage site mutant of F protein. This mutant is resistant to cleavage of functionally inactive F1 and F2 subunits by cellular trypsin-like proteases. Immunization with this mutant induced a mixed Th1/Th2 response, as demonstrated indirectly by production of IgG1 and IgG2a anti-Sendai virus antibodies *(1061)*.

Genetic immunization of neonates also induces a mixed Th1/Th2 response by circumventing deficient induction of Th1 cells during early life. We compared the cellular responses induced by immunization with a plasmid containing the influenza virus hemagglutinin (HA) gene in mice immunized as newborns or adults. DNA immunization protected both neonatal and adult mice from a challenge with a lethal dose of live influenza virus *(643)*. The immunization of adults elicited a Th1 response, whereas that of neonates elicited a mixed Th1/Th2 response. Similar results were obtained in newborn mice immunized with plasmids containing measles virus HA, Sendai virus nucleoprotein, or C fragment of tetanus toxin. In these experiments, the immunization of newborns with plasmids induced adult-like Th1 or mixed Th1/Th2 responses. These responses are characterized by production of IFN-γ by antigen-specific T cells. IgG2a was produced in mice immunized with measles virus HA plasmid, and both IgG1 and IgG2a was produced in mice immunized with a live recombinant canarypox vector expressing the same gene *(644)*.

The induction of Th1 responses by DNA immunization of neonates is related to CpG motifs contained in the plasmid. This was demonstrated by studying the response to hepatitis B virus in newborns. Mice immunized at 1, 3, 7, or 14 d after birth with hepatitis B surface antigen (HBsAg)-containing plasmid, HBsAg in combination with CpG oligonucleotide, or HBsAg in combination with CpG nucleotide and alum produced both IgG1 and IgG2a anti-hepatitis B virus antibodies. However, mice immunized with HBsAg and alum produced only IgG1 antibodies *(1062)*. These findings strongly suggest that the CpG motifs on plasmids that express various foreign genes may circumvent the biased Th2 response in neonates by stimulating the Th1 response. This effect is probably related to the activation of APCs following the binding of CpG to its corresponding Toll-like receptor. CpG binding to its receptor on APCs may trigger the IL-12 production that is required for the expansion of Th1 cells and the upregulation of costimulatory molecules such as CD40, CD80, and CD86. This concept is supported by data demonstrating that the immunization of newborn mice with plasmid that contains the antigen together with plasmid that bears IL-12 or IFN-γ genes enhanced the Th1 response *(1063)*.

In human newborns, the T-cell responses are also biased toward Th2, as illustrated by reduced IFN-γ production in cord blood lymphocytes stimulated with polyclonal activators *(1032,1064,1065)* that reach adult levels by age 12 mo *(1066)*. The defective Th1 response in neonates might be related to a lack

of CD45R0 memory cells in the cord blood and/or immature dendritic cells. It is well known that dendritic cells are required for activating naïve T cells. The reduced capacity of cord blood dendritic cells to stimulate Th1 responses may be related to reduced expression of costimulatory molecules; autosecretion of IL-10; and failure to produce IL-12, an IFN-γ-inducing cytokine, even after stimulation with lipopolysaccharide (LPS), which induces dendritic cell maturation *(1055)*. In contrast to adult dendritic cells, cord blood dendritic cells fail to adopt a mature phenotype following in vitro LPS stimulation, as evidenced by lack of upregulation of MHC class II molecules, reduced expression of CD25 and CD83, and minimally increased expression of CD86 *(1055)*. Autocrine synthesis of IL-10 may be another limiting factor. This is illustrated by increased production of tumor necrosis factor (TNF)-α and IL-12 and the capacity to activate Th1 cells subsequent to treatment of dendritic cells with anti-IL-10 neutralizing antibodies *(1067)*. The most striking difference between adult dendritic cells and cord blood dendritic cells entails the inability of cord blood dendritic cells to produce IL-12 upon in vitro LPS stimulation *(1065)*.

Human neonatal T cells have the ability to mount a Th1 response in certain conditions. Yu et al. *(1068)* have shown that in vitro stimulation of cord blood mononuclear cells with *Dermatophagoides pteronyssinus* extract produced significantly increased amounts of IFNγ and equal amounts of IL-4 compared to adult peripheral blood cells. This is associated with the upregulation of the T-bet transcription factor required for gene activation of Th1 cells, followed by increased expression of GATA-3. This result suggests that stimulation of cord blood lymphocytes may trigger the activation of Th1 cells associated with changes in the kinetics of T-bet/GATA-3 expression.

In vivo studies also demonstrated that Th1 responses could be induced in newborns immunized with BCG or in young infants infected with *Bordetella pertussis*. In adults, immunization with BCG induced a Th1 immune response. Study of the cytokines produced by T cells in infants immunized with BCG after birth showed that subsequent to in vitro stimulation with pumped derived protein (PPD), smallpox antigen 85 complex *(1069)*, and 10 kDa antigen, the T cells proliferated and produced IFN-γ but not IL-5 and IL-13, which are produced by Th2 cells. This is in contrast to T cells stimulated with phytohemagglutinin (PHA), which produced IFN-γ, IL-5, and IL-13 *(1068)*. Similar results were obtained in another study that showed T cells from children immunized with BCG after birth produced IFN-γ after stimulation with PPD and exhibited an increased frequency of IFNγ-producing cells similar to that observed in BCG-vaccinated adults *(1070)*. These studies clearly showed that neonatal BCG vaccination induces adult-like Th1 immune responses.

Induction of Th1 immune responses was also reported in 2-mo-old infants infected with *B. pertussis*. An increased production of IFN-γ and an increased

number of CD4 and CD8 IFN-γ-producing T cells were observed in lymphocytes obtained from acutely infected children following in vitro stimulation with *Bordetella* filamentous HA, pertussis toxin-specific antigens, or PHA *(1071)*. These results clearly show that lymphocytes from infants are mature and able to develop a Th1 response. The Th1 response induced by BCG and *Bordetella* is related to the activation of APCs by molecules of bacterial origin, such as peptidoglycan in the case of *Mycobacteria* or endotoxin in the case of *B. pertussis*, which bind to Toll-like receptors and activate the APC.

4. CTL RESPONSE IN NEONATES

CD8 CTLs play a major role in CMI responses against viruses and tumor cells. They recognize peptides derived from viral proteins and tumor-associated antigens presented by MHC class I molecules. It was long believed that, because of immaturity, neonatal T cells could not mount a CTL response against viruses, and, therefore, they could not kill infected cells or contribute to clearing the infected cells. Ensuing years have seen numerous findings that murine and human neonatal T cells can develop a CTL response in certain conditions.

We have studied the priming of CTLs in various stages of ontogeny with transfectoma cells that express a chimeric Ig heavy-chain gene bearing an influenza virus nucleoprotein (NP) peptide. The NP of influenza virus contains an epitope corresponding to amino acid residues 147-161 that is recognized by CD8 T cells in association with MHC class I K^d molecule. Through genetic engineering, we constructed a chimeric Ig molecule in which the CDR3 segment of the heavy chain was replaced with the NP147-161 peptide *(1072)*. The chimeric heavy-chain gene was transfected into SP/2 myeloma cells.

Study of CTL priming in adult mice showed that the NP-specific precursors can be expanded following immunization with NP peptide combined with Freund's complete adjuvant or with irradiated transfectoma cells bearing chimeric Ig heavy chain. Precursors could not be expanded with chimeric Ig-NP molecules or with SP/2 myeloma cells coated with NP peptide. In the same study, newborn mice were immunized on d 1, 3, and 5 after birth with 1 mg NP peptide, 150 μg Ig-NP, or 10^7 irradiated transfectoma cells. One month later, these mice were boosted with NP peptide in Freund's complete adjuvant, and 1 wk later, the lymphocytes were cultured with either NP peptide-coated or PR/8 influenza virus-infected cells. The CTL activity was then measured by using target cells coated with peptide. Our results showed that the immunization of neonates with the NP peptide or Ig-NP molecule failed to prime CTLs. In sharp contrast, mice injected at birth with transfectoma cells developed a cytotoxic response after in vitro secondary stimulation with NP peptide-coated spleen cells *(1073)*. These findings show that the generation of viral peptide from a

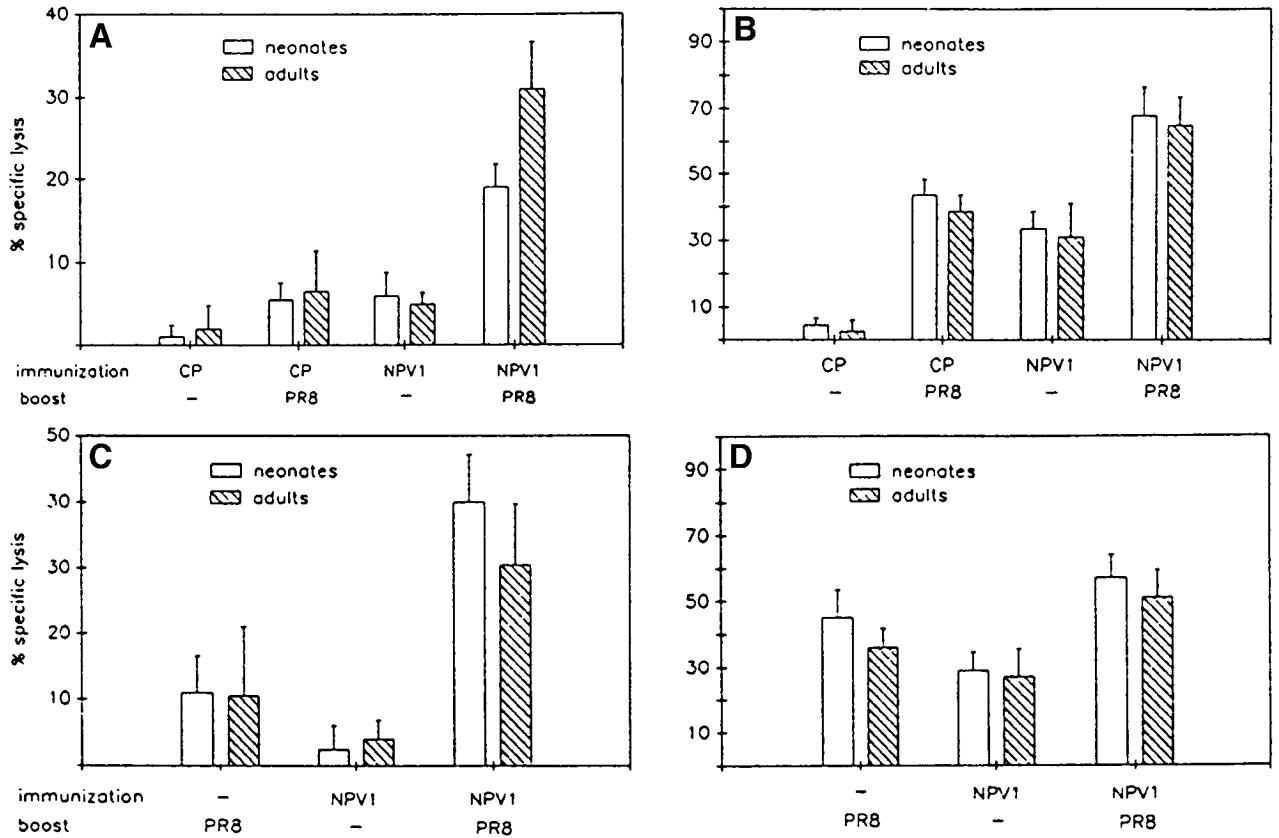


Fig. 40. Primary and secondary CTL response of mice immunized as neonates or adults with a plasmid containing influenza virus nucleoprotein gene. **A** and **C** are primary cytotoxic activity. **B** and **D** are secondary cytotoxic activity after immunization with empty plasmid (CP), plasmid containing nucleoprotein gene (NPVI), or PR8 influenza virus. (From Bot, et al. Dev Immunol 1998;5:197–210.)

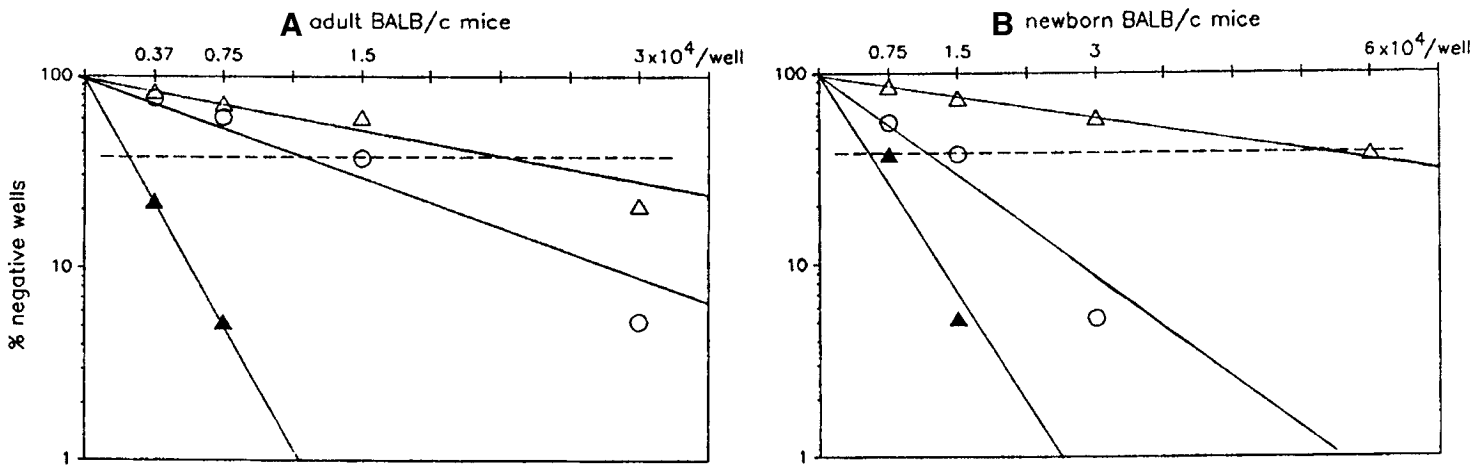
chimeric gene in an endogenous processing pathway efficiently primes neonatal precursors of NP-specific CTLs. Meanwhile, the soluble NP peptide and Ig-NP molecule failed to prime CTLs in neonates, as in the case of adults.

The priming of murine neonatal CD8 CTLs was also induced by immunizing mice after birth with low doses (0.3 or 1 PFU) but not with high doses (1000 PFU) of murine leukemia virus. CTL activity was detected 10–15 d after infection and persisted for at least 28 wk. The inability of neonates to develop CTL responses subsequent to immunization with a high dose results from a polarized Th2 response, which induces humoral immunity. This was clearly demonstrated by measuring the synthesis of IL-4 and IFN-γ. Whereas mice immunized after birth with a low dose of virus produced IFN-γ and low amounts of IL-4, those immunized with a high dose produced IL-4 but failed to synthesized IFN-γ *(1074)*.

Expansion of CD8 CTLs was also reported in mice immunized after birth with polyoma virus, which is a potent oncogenic mouse pathogen. Polyoma virus induced tumors in newborns of an inbred strain resistant to the virus. In the resistant strain, the CTLs specific for MT389 peptide, which is derived from a polyoma viral protein, dramatically and rapidly expanded during acute infection of neonates, reaching adult levels and leading to virus clearance. Although capable of generating MT389-specific CTLs, neonatal mice susceptible to polyoma virus infection cleared the virus at a markedly lower rate *(1075)*.

Efficient CTL immune responses can be induced in neonates by delivering the antigen by naked DNA. Our laboratory first demonstrated that newborn mice immunized with a plasmid-expressing NP of influenza virus (NPV1) developed a significant cytotoxic immunity comparable to that of adult mice immunized with the same dose of plasmid *(641)*. Comparison of primary and secondary CTL activity of mice immunized with NPV1 plasmid as newborns or adults and boosted with PR/8 influenza virus showed significantly higher NP-specific primary CTL activity than that of mice immunized with plasmid or virus alone. In contrast, increased secondary CTL activity was observed in mice immunized with NPV1 plasmid that was a little lower than that of mice immunized with NPV1 plasmid and boosted with PR/8 virus (Fig. 40). Increased CTL activity of mice primed with NPV1 plasmid and boosted with PR/8 virus resulted from an increased frequency of NP-specific CTL precursors, as illustrated in Fig. 41.

The priming of CTLs by NPV1 plasmid was independently assessed by measuring IFN-γ production by T cells from immunized mice stimulated in vitro with NP-peptide in the presence of APCs. Significantly higher amounts of IFN-γ were detected in the culture of T cells from mice primed with NPV1 plasmid and boosted with PR/8 virus than in culture from mice immunized



immunization	frequency	1/frequency	relative frequency
○ PR8	8.3×10^{-5}	1.2×10^{4}	1
△ NPV1	4.3×10^{-5}	2.3×10^{4}	0.5
▲ NPV1 and PR8	4.0×10^{-4}	2.5×10^{3}	4.8

immunization	frequency	1/frequency	relative frequency
○ PR8	8.3×10^{-5}	1.2×10^{4}	1
△ NPV1	1.9×10^{-5}	5.4×10^{4}	0.2
▲ NPV1 and PR8	2.0×10^{-4}	5.0×10^{3}	2.4

Fig. 41. Frequency of PR8 virus-specific pCTLs in spleens of mice immunized as adults (**A**) or neonates (**B**) with NPVI plasmid. The analysis of frequency of pCTL was carried out 4 wk after immunization with PR8 virus, NPVI plasmid, or both. (From Bot, et al. Dev Immunol 1998;5:197–210.)

Table 30
Effect of Immunization With NPVI Plasmid on Pulmonary Virus Titer and Survival After the Challenge With LD_{100} Live Influenza Virus

Mice	Immunization	Pulmonary virus titer d 3	d 7	d 16	Survival
Adult					
1 mo	Saline	4.6±0.5	3.8±0.1	NS	0%
after immunization	PR8 virus	0	0	ND	100%
	Control plasmid	4.8±0.1	3.7±0.5	NS	0%
	NPVI plasmid	4.0±0.3	0.9±1.5	0	80%
3 mo	NPVI plasmid	4.8±0.1	0.2±0.2	0	65%
after immunization					
Newborn					
1 mo	Control plasmid	5.9±0	4.6±0.2	NS	0%
after immunization	NPVI plasmid	4.5±1.2	1.2±2.1	0	30%
3 mo	NPVI plasmid	4.1±0.5	0.9±1.2	0	70%
after immunization					

Adult mice were immunized with 3×39 μg control or NPVI plasmid at 3-wk intervals in the anterior tibial muscle of the right leg. A group of adult mice were immunized i.p. with PR8 virus 7 d before challenge.

Newborn mice were immunized with 3×30 μg with control or NPVI plasmid 1, 3, and 6 d after birth in the right gluteal muscle.

Both groups of mice were challenged 1 or 3 mo after completion of immunization with aerosols containing a LD_{100} dose (1.5×10^4 $TCID_{50}$) of PR8 influenza virus.

Pulmonary virus titer was measured by chicken red blood hemagglutination after 48 h incubation of MDCK cells with serial dilutions of lung homogenate. The results are expressed as mean ± SD of $\log_{10}$ $TCID_{50}$ measured individually for each animal in a group of three mice.

NS, no survivors; ND, not done.

with virus alone or with empty plasmid (control). Decreased pulmonary viral titers and increased survival after challenge with live virus (LD_{100}) was observed in mice immunized as newborns or adults with NPV1 plasmid (Table 30). It is noteworthy that immunization with NPV1 plasmid induced CTL responses against two different influenza type A viruses. This effect might occur because influenza type A viruses that differ in the structure of the HA gene share an identical NP gene *(1076)*.

A robust adult-like CD8 CTL protective immunity was induced by the immunization of newborn mice with plasmid containing DNA clones of murine leukemia virus *(1077)*, NP gene of LCMV *(1078,1079)*, and HA of measles virus *(644)*. In all experiments, the CTL immunity induced by genetic immunization was long-lived and comparable to that induced in adults. These findings

clearly demonstrate that DNA-based immunization of mice circumvents the ontogenic delay in development of CTL precursors and may prove an effective and safe strategy for the development of vaccines for infants.

Efficient induction of CD8 CTL immunity by genetic immunization may be related to in vivo transfection of dendritic cells by gene gun *(91)* or subcutaneous *(89)* immunization. Among various types of APCs, the dendritic cells are the most efficient in initiating the immune response of naïve T cells. Bot et al. *(89)* demonstrated that at the site of NPV1 plasmid injection, dendritic cells are transfected. Furthermore, adoptive transfer experiments showed that the expansion of NP-specific CTL precursors with class II^+ dendritic cell cells required 10 times less cells than with class II^- cells.

It is known that generating memory CTL cells requires the presence of antigen *(1080)*. Long-lived responses induced by genetic immunization are probably related to the persistence of plasmid as episomes, allowing for continuous priming of newly emerged T cells from the thymus and for generation of memory cells during postnatal life. As an example, in humans, the expansion of HIV-1-specific CTLs was observed in an infant infected *in utero (1081)* and in infants born to seropositive mothers *(1082)*. The HIV-1-specific CTL activity was detected from age 3 mo to 5 yr in the infant infected *in utero* but only during late infancy in some children born to seropositive mothers with overt HIV-1 infection.

Secondary CTL responses were also observed in infants with acute respiratory syncytial virus (RSV) infections *(1083)* and in infants after natural infection or immunization with live influenza A virus *(1084)*. It is noteworthy that no influenza virus-specific CTL activity was detected in infants after immunization with cold-adapted or inactivated influenza vaccines. Failure to generate influenza virus-specific CTLs after immunization with inactivated vaccine is expected, because internalized killed virus is processed in the exogenous pathway, which does not generate peptide able to bind class I molecules. Lack of CTL activity in infants vaccinated with cold-adapted virus may be related to the lower replication rate of virus in vivo. In addition, limited replication in the nasal cavity but not in the lung might decrease the chances of activating CTLs by virus-infected pulmonary macrophages or epithelial cells.

In conclusion, the information presented in this chapter strongly supports the idea that the paradigm of unresponsive neonatal T cells has restricted validity. Neonatal lymphocytes can mount effector and memory cells in certain conditions related to antigen dose, delivery platform of the antigen, and the cytokine microenvironment, particularly the pattern of cytokines secreted by APCs induced by microbial agents. When adequate cytokines are present, the Th2 dominance of the neonatal response can be switched to Th1 responses, mediating defensive reactions against intracellular pathogens.