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Review

Genetic immunization of neonates

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Abstract

The vaccination of neonates is generally difficult due to immaturity of the immune system, higher susceptibility to tolerance and potential negative interference of maternal antibodies. Studies carried out in rodents and non-human primates showed that plasmid vaccines expressing microbial antigens, rather than inducing tolerance, triggered significant humoral and cellular immunity with a Th1 component. The ability of bacterial CpG motifs to activate immature antigen-presenting cells is critical for the neonatal immunogenicity of DNA vaccines. In addition, the endogenous production of antigen subsequent to transfection of antigen-presenting cells may explain the lack of inhibition by maternal antibodies of cellular responses. Together, these features make the plasmid vaccines an appealing strategy to prime immune responses against foreign pathogens, during early life. In combination with subsequent boosting using conventional vaccines, DNA vaccine-based regimens may provide a qualitatively superior immunity against microbes. Thorough understanding of immunomodulatory properties of plasmid-vectors may extend their use for early prophylaxis of inflammatory disorders.

Keywords: DNA vaccination; Newborns; CpG motifs; Tolerance

1. Induction of anti-microbial responses by neonatal DNA vaccination

The potential of DNA vaccines to trigger cytotoxic T lymphocyte (CTL) responses stimulated the research in various models of infectious diseases, particularly those caused by viruses. Since neonates have a depressed immunity and limited immune memory [1] that make them susceptible to serious infections, a natural target age for improved vaccines would be that corresponding to neonatal stage.

The induction of tolerance became a paradigm for neonatal responsiveness, given early observations that newborn mice injected with high numbers of allogenic hematopoietic cells failed to reject allografts [2]. Recent studies describing a dose–effect relationship between neonatal responsiveness and amount of antigen argued that the limited numbers rather than immaturity of lymphocytes may be the cause of neonatal susceptibility to tolerance [3]. However, additional studies debated the hypothesis that the peculiar neonatal responsiveness is just a matter of high zone tolerance due to low number of mature lymphocytes. Thus, the immature B cells which are predominant in the periphery of neonates display characteristic features which may explain their susceptibility to tolerance: in the absence of surface IgD, the antigen-triggered signaling pathway is not coupled to the inositol-phospholipid cascade [4] and the expression of *src*-family tyrosine kinases is reduced [5]. In addition, the relatively few mature B cells in the periphery of human neonates express lower levels of membrane CD40. This, together with decreased expression of CD40L on T cells, may account for the low production of IgG, IgA and IgE isotypes during the early period of life [6]. On the other hand, impaired CD40-CD40L triggered expression of IL-12 may explain the tendency of neonates to develop Th2 responses [7]. Partial restoration of cytokine-producing ability of neonatal T cells by addition of anti-CD28 mAb elegantly illustrated the concept of limited co-stimulation during the neonatal interval [8]. At the origin of the limited co-stimulation may be the immaturity of antigen-presenting cells (APC) during the neonatal interval [9,10]. Recently, the importance of complement system in the generation of adaptative immunity and particularly the B-cell response, has been characterized [11]. C3 components may contribute to the B-cell recognition of antigens, via co-engagement of antigen and complement receptors on the membrane. In addition, the complement receptors expressed on the follicular–dendritic cells may prolong the exposure of antigen in the germinal centers, where affinity maturation of antibodies is being triggered. Since during early life the complement system is not fully matured and the levels of C3 components

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Table 1
Neonatal immunization with DNA expressing microbial antigens

Microbe	Antigen	Species	Immune response	Reference
Influenza virus	NP, HA + IFNγ or IL-12	Mouse	Abs, CTL	[16,30–32,41,50,61]
		Non-human primate	Th, Abs	[27,28]
Plasmodium yoelii	CSP	Mouse	Tolerance	[15]
	CSP+GM-CSF	Mouse	Abs, CTL	[59]
Pseudorabies virus	gD glycoprotein	Pig	Abs	[17]
Rabies virus	Glyco protein	Mouse	Abs, Th	[18]
Hepatitis B virus	HBsAg	Non-human primate	Abs	[26]
Murine retrovirus	Cas-Br-M antigen	Mouse	CTL	[22]
HIV	Env	Non-human primate	Abs	[25]
	Gag-Pol			
Measles virus	Hemagglutinin	Mouse	Abs, Th	[20]
Sendai virus	NP	Mouse	Abs, CTL	[20]
Clostridium tetanii	C fragment of tetanus toxoid	Mouse	Abs, Th	[20]
Herpes simplex	gB	Mouse	Abs, Th	[19]
LCMV	NP	Mouse	CTL	[24,35]
Respiratory syncitial virus	F antigen	Mouse	Abs, CTL	[23]
Bovine herpesvirus	gD	Sheep	Abs, Th	[65]

are limited [12], this may contribute to the decreased and altered immunity induced by neonatal vaccines. Interestingly, coupling the hemagglutinin of influenza virus and the C3d fragment in the same open reading frame resulted in an increased B-cell response in the context of DNA vaccination [13]. In addition, a differential regulation of immune response during the neonatal stage by endogenous corticosteroids [14] may alter the immune profile to foreign antigens. Altogether, these studies suggested that intricate qualitative and quantitative factors are responsible for the characteristic neonatal responsiveness.

Conventional vaccines display some limitations in the early age group: poor immunogenicity in case of killed or subunit vaccines, potential side effects for live vaccines, or inhibition by maternal antibodies in all these circumstances. These factors promoted a substantial number of studies during the last decade in the area of neonatal or early DNA vaccination, with over 20 published studies. Indirectly, this contributed to a better understanding of the process of maturation of the immune system.

1.1. Profile and magnitude of immune response

Numerous groups have addressed the nature of immunity triggered by DNA vaccines given shortly after birth. Based on previous knowledge, it was not easily to predict the outcome of exposure to low amounts of antigen for prolonged intervals during the neonatal stage. However, various protocols of neonatal inoculation of DNA vectors expressing a wide range of microbial antigens into mice or non-human primates resulted in induction of humoral, Th and CTL responses rather than unresponsiveness (Table 1), with one notable exception [15]. Our studies, for the first time, showed the induction of CTL able to mediate influenza virus clearance, by neonatal immunization of mice with a plasmid encoding the nucleoprotein (NP) of influenza virus [16]. At the same time, another group reported the induction of weak antibody responses rather than tolerance, by immunization of piglets with plasmid expressing the gD antigen of pseudo-rabies [17]. Soon, reports regarding neonatal DNA vaccination against the rabies virus glycoprotein [18], herpes simplex gB antigen [19], measles virus hemagglutinin (HA), the C fragment of tetanus toxin [20] and the L protein of hepadnavirus [21] showed induction of humoral responses in mice and other species. These reports were mirrored by observations regarding the induction of specific CTL against the Cas-Br-M antigen of a murine retrovirus [22], the NP of Sendai virus [23] and the NP of lymphocytic choriomeningitis virus (LCMV) [24]. More recently, the immunogenicity in terms of humoral response of DNA vaccines against HIV antigens [25], the hepatitis B virus (HBV) surface antigen [26] and the HA of influenza virus [27,28], was extended to newborn non-human primates.

Qualitative and quantitative differences do exist between the immune responses elicited by neonatal versus adult inoculation of DNA. The Th profile elicited by neonatal DNA immunization comprises an important Th1 component [29], resembling the adult response and generally contrasting with the neonatal immunity triggered by conventional antigens. However, there is a significant Th2 component as well, explaining the induction of a mixed IgG1/IgG2a humoral response [20,29]. An independent group recently confirmed the tendency of mice immunized as neonates with a plasmid expressing the HA of influenza virus to mount mixed Th1/Th2 responses [30]. A bias towards Th1 immunity could be provided via co-injection of IL-12 or IFN-γ-producing plasmids, observation that elegantly pinpointed these cytokines as limiting factors during the neonatal period [31].

Subsequent boost with virus or infectious challenge of mice immunized at birth with DNA expressing HA or NP of influenza virus, was followed by a substantial increase in antibody titers and expansion of Th and CTL [16,29,32].

However, quantitatively, the expansion of CTL was relatively reduced compared to that noted in adult mice [32]. Nevertheless, CTL induced by neonatal DNA vaccination against the NP of influenza virus were of Tc1 phenotype and mediated the clearance of homologous or heterologous virus if the pCTL frequency was above a certain threshold (roughly 10^3 specific precursors/spleen). The kinetics of CTL induction was slower in neonates as compared to adults, probably reflecting the immaturity of the T-cell repertoire. Quantitative differences were noted in the case of antibody titers as well: whereas the titers of neutralizing antibodies persisted a longer time at high levels in adults immunized with plasmid expressing the HA of influenza virus, they declined more rapidly in mice immunized as neonates [29].

Despite the fact that the magnitude of the immune response elicited by neonatal DNA immunization was decreased when compared to adult DNA immunization, a number of studies reported significant protection conferred by neonatal DNA vaccines against infectious challenge carried out during adulthood. Such protection relied on CTL in the case of the NP antigen of influenza virus [16,32], the Cas-Br-M antigen of a murine retrovirus [22] and the NP of LCMV [24]. Furthermore, protective Th-dependent humoral immunity was elicited by neonatal immunization with plasmids expressing the glycoprotein of rabies virus [17], the HBs antigen [26] and the gB antigen of herpes simplex [19]. No protection was noted after DNA immunization of piglets against pseudo-rabies [17]. Our studies showed a dramatic improvement in the protection conferred by neonatal DNA immunization, when mice were inoculated with plasmid mixtures expressing dominant B, Th and CTL epitopes [33].

By measuring the kinetics of virus-specific pCTL number in the spleen of mice immunized as neonates with DNA expressing the NP of a type A influenza virus, we have shown that the memory pool expands between 1 and 3 months [32]. This indirectly but strongly suggested that the exposure to antigen continued after 1 month and that during the first month of life, the reduced T-cell repertoire is a limiting factor. However, the exposure of lymphocytes to antigens expressed by neonatal DNA vaccines occurs very early since mice immunized with NP-expressing plasmid and boosted at the age of 3 weeks with virus displayed substantial immunity reminiscent of priming [16,32]. Secondly, mice immunized at birth with a plasmid expressing the HA of a type A influenza virus mounted mixed Th1/Th2 responses, rather than Th1 response like 4 week old mice [29]. All these data strongly suggest that while exposure of lymphocytes to antigens expressed by neonatal DNA vaccines begins before the age of 2 weeks, it may last until after the age of 1 month. However, the data based on prime/boost regimens with DNA vaccines during the neonatal window suggested that without necessarily inducing unresponsiveness, early inoculations are less effective in inducing protective immunity [33]. This observation may reflect differences in the antigen expression regarding cell type and duration, depending on the precise developmental stage at immunization.

One may take advantage of the improved quality of the T-cell profile triggered subsequent to neonatal DNA vaccination, by boosting with conventional vaccines in order to further amplify the immune response. This is based on the commitment of differentiated lymphocytes to maintain their profile upon subsequent antigen encounter and has been illustrated in a model of immunization against the respiratory syncytial virus [23]. A variant to this protocol may consist in co-administration of conventional vaccines together with CpG oligonucleotides: indeed, surprisingly, co-formulation of a prototype protein vaccine together with CpG immunostimmulatory motifs in alum resulted in induction of substantial immunity as well as CTL responses in newborn mice [34]. Besides the mechanistic implications described below, this type of strategy would facilitate a more rapid application in the area of vaccines since it circumvents the requirement to develop antigen-expressing plasmids.

1.2. Memory

Based on long-time persistence of plasmids at the site of inoculation reported by numerous groups, it has been assumed that the immune memory generated by DNA vaccines would last accordingly. Due to the fact that first, immune memory may persist in the absence of antigen and secondly, that persistence of plasmid may not necessarily reflect continuous exposure to antigen, it may be difficult to predict the answer to this question.

While earlier neonatal DNA vaccination studies focused on the concept of immunogenicity/unresponsiveness and the induced immune profile, more recent work systematically analyzed the magnitude and nature of immune memory [35]. By employing a sensitive technique, consisting of intracellular cytokine staining of T cells restimulated ex vivo for a brief period with peptide, the authors showed a remarkable life-long persistence of T memory cells subsequent to neonatal DNA vaccination with NP of LCMV. Despite the equilibrated Th1/Th2 profile suggested by the antibody isotype pattern, class I-restricted $CD8^+$ T cells expressing perforin and capable of rapidly up-regulating IFN-γ production, persisted for 1 year at a frequency of 0.5–1% of total $CD8^+$ T cells. This immune profile correlated with the protection against viral challenge, manifested by reduction of virus titers in tissue. The results of Hassett et al. contrast significantly with our data in the influenza NP system: we showed a sharp decrease in the frequency of specific CTL measured by limiting dilution analysis, beyond the age of six months, in mice immunized as newborns [32]. This was paralleled by loss of protection against lethal challenge with influenza virus [32]. The discrepancy between the results of these studies may be due to the plasmid vectors employed, differences in the size of the repertoire of

naïve, specific T cells; presence of dominant helper epitopes; and/or intrinsic differences between the read-out methods and their significance in these cases. For example, the size of the subpopulation of specific $CD8^+$ T cells that produces cytokines, may be larger than the pool of cells endowed with cytotoxic properties as suggested by recent studies [36].

We addressed the generation of humoral and cellular memory in non-human primates immunized with a prototype DNA vaccine as newborns. In pilot studies, we demonstrated that co-administration of NP and HA-expressing plasmids to newborn mice resulted in a synergistic protective effect against infectious challenge carried out beyond the neonatal window (1–3 months) [33]. We tested whether inoculation of such plasmids into newborn baboons (at the age of 1 d and boosted on day 14 and 28), is followed by induction of humoral and cellular responses that persist beyond infancy. We determined that higher doses of plasmid (1 mg/plasmid/dose) result in induction of persisting titers of virus-specific IgG and most importantly, in humoral and cellular memory revealed subsequent to boost with virus at the age of 1.5 years [37]. Despite the modest neutralizing titers of antibodies triggered by plasmid vaccination alone, after boost, the endpoint titers were substantial (at least four-fold higher than those of non-primed controls, in the order of thousands). This suggested that a prime/boost strategy may be a better way to take practical advantage of our findings. Namely, priming with DNA vaccine during the neonatal window followed by conventional boost during infancy or early childhood (live attenuated virus via respiratory tract in the case of anti-influenza vaccination), may be the most optimal/safest strategy to raise anti-influenza, broad immunity during early life.

1.3. Neonatal vaccination and effect of maternal antibodies

Maternal antibodies are transmitted via the placenta or milk to offspring and they confer passive immunity to microbes that infect relatively immunodepressed neonates and infants. A prerequisite for the effectiveness of passive immunity conferred by maternal antibodies is the conservation of antigens associated with strains that elicited maternal immunity and strains that infect the offspring. Viruses that rapidly mutate their dominant B-cell epitopes, like the influenza virus HA determinants, may have limited potential in generating effective maternal immunity. However, there is significant likelihood that maternal antibodies recognize B-cell epitopes of strains in circulation, including those incorporated in contemporary vaccines, based on conventional antigens. Thus, the neonatal vaccination with such vaccines may face maternal antibodies that bind to surface epitopes and rapidly clear the vaccine. The worst-case scenario may occur when maternal antibodies instead of recognizing neutralizing epitopes that mutate during drift variation, bind to non-protective B-cell epitopes and thus interfere with the efficiency of vaccine but have limited ability to neutralize infectious virus. As another example, measles virus that displays much less B-cell epitope variability may pose similar obstacles regarding neonatal vaccination and maternal immunity. Namely, long-term persisting antibodies triggered by natural infection or live attenuated vaccination may be transmitted to offspring, where they may interfere with the immunogenicity of the vaccine. Numerous observations documented that maternal antibodies may actually inhibit the protective immunity triggered by vaccines early in life. Such data were generated in the case of measles virus [38], respiratory syncytial virus [39], equine influenza virus [40], malaria [41], rabies virus [42] and others. This is in fact one of the main reasons for the late administration of certain vaccines such as the measles vaccine, after the maternal antibodies decayed in the circulation.

Although during the last two decades different models were proposed to explain the phenomenon of maternal interference—including active suppression by regulatory lymphocytes—more recently it has been proposed that the binding of the antibodies to the vaccine antigens caused this effect. Thus, the formed immune complexes may preclude the infection in the case of live attenuated vaccines or clear the antigen in the case of subunit vaccines, before engagement of B-cell receptors. The antigen trapped in immune complexes is internalized via Fc receptors (FcR) into non-professional APC, where it can be completely degraded in endolysosomes (Fig. 1A). Resulting peptides may or may not be exocytosed or displayed in the context of nascent MHC class II molecules, upon displacement of the invariant chain (Ii) in the CII vesicles. Since degradation of proteins in endolysosomes greatly precludes preservation of conformational B-cell epitopes, the spectrum of antibodies to exocytosed fragments is greatly narrowed to those recognizing linear epitopes. Thus, due to the conformational nature of protective epitopes such as receptor-binding motifs, this results in unfavorable conditions for induction of protective antibodies in the context of maternal immunity. In addition to the interference with the recognition of neutralizing epitopes, immune complexes may preclude or modify generation and recognition of MHC class II-associated epitopes. In vitro studies showed that virus-specific polyclonal or monoclonal antibodies inhibited the presentation of a major viral MHC class II-restricted epitope by professional APC, when such APC were in vitro pulsed with immune complexes [43]. This was postulated to occur by redirecting the immune complexes to lysosomal compartments without endosomal transfer, although one cannot rule out differential processing in endosomes with generation of distinct peptides. In addition, we cannot rule out that the identity and degree of activation of APC involved in clearance and processing of immune complexes are different from those of APC subsets involved in handling of antigens in the absence of specific antibodies. As showed

recently, cross-linking of FcγR expressed on APC resulted in induction of IL-10, a potent immunosuppressor molecule [44]. Using FcγR knock-out mice, it has been shown that FcγRI in particular were responsible for triggering IL-10 production upon receptor cross-linking (H. Zaghouani, in preparation). IL-10 triggers down-regulation of expression of MHC class II and of co-stimulatory molecules on APC [45] and thus may prevent activation of Th cells. At this point, it is unclear whether the lack of antigen exposure to B-cell receptors or the impaired Th activation, are the limiting factors responsible for negative interference by maternal antibodies. Finally, the reduced activation of CTL by live vaccines in the presence of maternal antibodies may be easily explained by prevention of cellular infection (Fig. 1A). It is not clear, however, why immune complexes containing viral antigens and maternal immunoglobulins are devoid of the ability to induce class I-restricted immunity in light of recent evidence to the contrary [46]. One explanation may be that the magnitude of the CTL response is greatly diminished but not abrogated in the presence of maternal immunity.

In view of potential interference between maternal immunity and neonatal or infant vaccination, much attention was given to DNA vaccines. The hypothesis was that specific maternal antibodies, unable to bind to the vaccine vector, do not interfere with the immunity induced by DNA vaccines. Studies have been initiated in various experimental models and a certain pattern begins to emerge. As in the case of conventional vaccines, it seems that the maternal antibodies negatively interfere with the humoral response to antigens expressed by DNA vaccines. Such results were reported in different experimental models of pseudo-rabies [17], measles virus, tetanus toxoid and influenza virus [47–49]. Using an experimental system that comprised CB F1(axb) mice bearing C_Ha and b allotypes, born from mothers of IgC_H^a allotype, it was demonstrated that either virus or DNA immunization of neonates failed to mount antibody responses in the presence of maternal antibodies [50]. Maternal versus endogenous antibodies were distinguished using reagents specific for allotypes of IgC_H. There were two interesting findings in this study that suggest an effect of maternal antibodies beyond passive clearance of immunogens during the neonatal window: first, the inhibitory effect in pups persisted beyond the decay of maternal antibodies. Secondly, there was a complex effect of maternal antibodies on the clonotype repertoire of B cells reactive to influenza virus epitopes, consisting of a broadened reactivity pattern. These findings may suggest a complex regulation of the developing B-cell repertoire via internal image antibodies, elicited by maternal immunoglobulins. In contrast, the cellular immunity induced by neonatal DNA vaccination was not affected by maternal antibodies transmitted from dams to offspring.

A more heterogeneous pattern emerged regarding the inhibition of T-cell responses by maternal antibodies. An interesting study approaching this issue in the system of

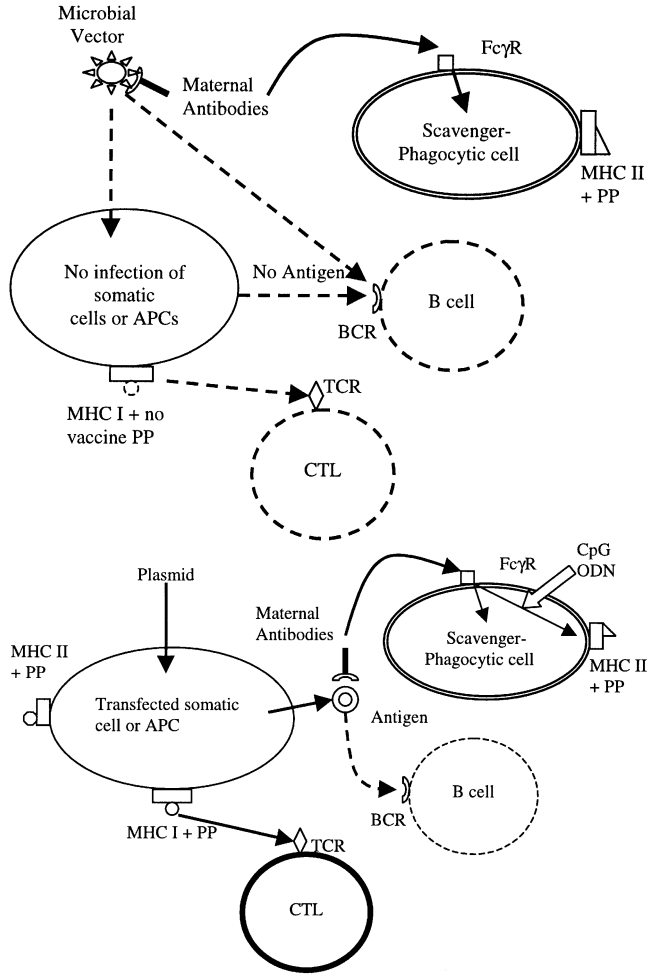

Fig. 1. A proposed model explaining the differential impact of maternal immunity on the immune response subsequent to neonatal immunization with conventional (A) or DNA (B) vaccines. (A) Conventional vaccines such as live attenuated are bound by maternal antibodies, interfere with infection of permissive cells and redirect the opsonized vaccine to phagocytic cells where degradation occurs in the endolysosomal compartment. Peptides generated may be presented in context of MHC class II molecules; however, the stimulation of antigen-reactive B cells and generation of class I-restricted epitopes is largely prevented in the absence of exposed antigen and cellular (abortive or replicative) infection, respectively. (B) In contrast, maternal antibodies do not bind to plasmid vaccine. Thus, generation of class I-restricted epitopes and activation of CTL is not inhibited. In addition, various mechanisms such as transport of peptides from cytoplasm to endolysosomes of in vivo transfected APC (left), or CpG-activation of APC that internalize immune complexes (right), may be responsible for generation of Th immunity to neonatal DNA vaccines in the presence of maternal antibody. Another possibility (not shown in the diagram) is shielding of B-cell epitopes by heat shock proteins during antigen transfer between in vivo transfected cells and APC. Finally, the activation of B cells may still be inhibited due to prevention of engagement of B-cell receptors.

murine herpes simplex infection showed that maternal antibodies did not impair the Th response elicited by neonatal DNA vaccine expressing the gB antigen [19]. In contrast, the response elicited by conventional vaccine was impaired. Interestingly, the antibody response triggered by neonatal DNA immunization was not inhibited by maternal antibodies in this particular situation. The lack of inhibition

of humoral response may be due to relatively low titers of maternal antibodies or conversely, to a mismatch between the fine specificity of antibodies elicited by the conventional antigen and epitopes displayed on the antigen expressed by the DNA vector, as other studies suggested as well [51]. This is indirectly supported by the findings of Mor et al. [15] that differential B-cell epitopes are displayed in the context of neonatal DNA vaccination and immunization with protein, respectively. Subsequent to DNA vaccination, the microbial antigens may be expressed by a distinct set of cells compared to the case of natural infection or conventional vaccination, particularly in parasitic and bacterial antigens. Thus, differential post-translational modifications may account for display of different B-cell epitopes in DNA or conventional vaccination, or microbial infection.

The lack of inhibition of T-cell responses generated by neonatal vaccines in the context of maternal antibodies was confirmed in the systems of measles virus, tetanus toxoid and influenza virus [47–50]. In measles virus, significant T-cell responses were obtained even subsequent to virus immunization in the context of maternal antibodies. Interestingly, CTL immunity induced by neonatal DNA vaccination with the NP of LCMV in the context of maternal antibodies, was not impaired [24]. We noted similar lack of inhibition by maternal antibodies of CTL response induced by neonatal DNA immunization with a plasmid expressing the NP of a type A influenza virus (Bot et al., not published).

Thus, in neonatal DNA vaccination, the rule seems to be that maternal antibodies might affect B-cell rather than T-cell immunity. Furthermore, DNA vaccines seem to perform better than conventional vaccines, from this point of view. This pattern is in agreement with the mechanisms that govern the immunity triggered by DNA vaccines. In humoral immunity, maternal antibodies may bind and scavenge antigens released by in vivo transfected cells, thus precluding the stimulation of a B-cell response. Circulating antigen is thought to be required for proper engagement of Ig receptors and activation of B cells. In contrast, in vivo transfected APC might directly present the resulting epitopes to class I-restricted CTL via the endogenous pathway of processing. Thus, maternal antibodies would not interfere with this process. Furthermore, if the transfer of epitopes occurs in a poorly antigenic form, the induction of CTL responses by cross-priming may not be affected in the context of maternal antibodies. Regarding the generation of Th responses, specific antibodies may display complex effects on immunogenicity of class II-restricted epitopes generated via the conventional exogenous pathway, ranging from inhibition to stimulation [52,53]. It is possible that a non-classical endogenous pathway of processing and presentation of class II-restricted epitopes may occur in the in vivo transfected APC [54,55], eluding the inhibitory effect of maternal antibodies. Clearly, the difference in the susceptibilities of B- and T-cell responses to inhibition by maternal antibodies pinpoints the particularities regarding the mechanisms of B- and T-cell priming.

This model is apparently challenged by recent discoveries and a few observations from the past: if cross-priming is a predominant mechanism for generation of Th and CTL responses in DNA-vaccinated organisms, then one would expect an inhibitory effect of maternal antibodies, similar to that noted in conventional vaccination. Secondly, one would predict that CpG-co-formulated conventional vaccines are inhibited by maternal antibodies. This was in fact not true, as shown in a recently published study by Weeratna et al. [56]. This study demonstrated that substantial CTL responses could be triggered by HBsAg formulated in alum with CpG ODN in newborn mice, in the presence of maternal antibodies. In fact, a previous study carried out in a viral model challenged the inhibitory role of passive immunity relative to the generation of CTL responses subsequent to live-virus immunization [57]. An alternative model emerges: thus, instead of bypassing the clearing effect of maternal antibodies, DNA vaccines via CpG immunostimulatory motifs, may activate APC to a level where they can effectively process immune complexes and present class I- and II-restricted epitopes in an immunogenic form. In contrast, non-CpG activated APC would largely fail to carry out this function, explaining the results with conventional vaccines. This model is not necessarily contradictory to the one described above, but adds an additional layer of complexity to it (Fig. 1).

While this model awaits confirmation or rejection, it would be interesting to extend to other microbial systems the studies regarding the influence of maternal antibodies on the immunity generated by various types of neonatal vaccines, and particularly, to address the effect on protection. The effect on protection would likely depend on the relative role of humoral versus cellular immunity in the defense against the pathogen considered.

2. Mechanisms of neonatal immune responsiveness to DNA vaccines

The studies on immunity induced by DNA vaccines in neonates bear implications on neonatal responsiveness in general. Thus, the observation that DNA vaccines are immunogenic rather than tolerogenic when given to newborns defines another circumstance of neonatal responsiveness. The immunity conferred by neonatal DNA vaccination closely resembles that generated in adults by DNA or live vector vaccination, with subtle differences regarding the magnitude and Th profile of the immune response. This is remarkable, taking into account the difficulties of inducing protective immunity by neonatal inoculation with conventional vaccines. Together with other observations regarding successful induction of immune response in neonates, the studies on neonatal DNA vaccination questioned the paradigm of neonatal tolerance as a critical determinant for self/non-self discrimination and promoted instead the well-accepted “danger model”.

Studies aimed at assessing the factors responsible for immunity to neonatal DNA vaccines pointed out the role of immunostimulatory CpG motifs. Indirectly, this revealed that co-stimulation is generally limiting during the neonatal stage and secondly, that CpG motifs may circumvent to a certain extent this defect by activating the immature neonatal APC. Co-inoculation of CpG motifs together with alum-formulated HBV surface antigen into newborn mice was followed by induction of immune response, similar to that obtained by DNA immunization [34]. In fact, the magnitude of immune response was higher in the case of CpG-formulated antigen, suggesting that the quantity of antigen is a limiting factor in neonatal DNA immunization. Interestingly, co-inoculation of CpG motifs allowed the generation of significant CTL responses to the protein, indicating that cross-priming can occur in the neonatal context. Similar evidence regarding the role of CpG oligonucleotides in neonatal DNA vaccination was recently obtained in a model of measles virus ([58]; C.A. Siegrist, unpublished). Thus, the CpG immunostimulatory motifs may help CTL induction by cross-priming and generation of Th1 responses subsequent to stimulation of neonatal APC. Such events may occur by triggering the IL-12 production, up-regulation of co-stimulatory molecules such as B7.1, B7.2, CD40 and by activating non-classical MHC class I-presentation pathways in APC. Interestingly, the concept of neonatal susceptibility to high zone tolerance was challenged by the observation that neonatal immunization with protein formulated with CpG oligonucleotides triggered enhanced rather than decreased responses or immune tolerance. It is possible that activation of additional professional APC may increase the threshold dose associated with high zone tolerance in neonates. A recent study pointed out as important limiting factor the reduced number of professional APC in neonates: co-administration of plasmids expressing GM-CSF and the circumsporozoite protein (CSP) into newborn mice triggered immunity rather than tolerance [59], that was previously achieved with the CSP-plasmid alone [15]. Since GM-CSF has a known effect on the differentiation and activation of dendritic cells, this result indicates their involvement in the response triggered by neonatal DNA vaccines and provides clues regarding how such vaccines may be improved. Supporting this model, co-administration of IL-12 or IFN-γ expressing plasmids together with DNA vaccine to newborn mice, optimized the Th1 component of the immune response [31].

In conclusion, the recruitment and activation of professional APC by immune stimulatory CpG motifs circumvents their limiting number and immaturity in neonates. Furthermore, continuous antigen production in low amounts, while decreasing the likelihood of tolerance, may contribute to the priming of functional T and B cells in the periphery during the neonatal stage. The most important limiting factors associated with neonatal responsiveness are the decreased GM-CSF, IL-12 and IFN-γ production by natural immune cells and the limited size of the immune repertoire. Consequently, early immunization with proper adjuvants may address both of these limiting factors resulting in feasible, neonatal vaccination strategies.

3. Down-regulation or deviation of immune responses by neonatal DNA vaccination

In view of the numerous findings that neonatal DNA vaccination triggers substantial immune responses, the use of plasmid expression vectors to reduce immunity may appear counter-intuitive. Nevertheless, there is accumulating evidence that plasmid vaccination may be used to modulate immune responses.

An exception from the rule of immunogenicity of neonatal DNA vaccines was reported in mice inoculated with a plasmid expressing the CSP of *Plasmodium yoelii* [15]. Namely, newborn mice immunized with that plasmid mounted long-lasting tolerance that selectively affected certain B- and T-cell epitopes on the circumsporozoite antigen. The responsiveness to epitopes normally recognized following protein immunization remained unaffected. In contrast, the adult mice were strongly immunized by this plasmid [15]. Whereas the reason for the discrepancy between this observation and the other studies on neonatal DNA vaccination remains elusive, this result should be complemented with the recent finding that neonatal DNA immunization with the same circumsporozoite antigen induced a rather peculiar $CD8^+$ bystander-suppressor population of T cells [60]. Thus, besides the natural tendency of the neonatal immune system to tolerance, certain properties of this antigen or expression vector may be critical for the noted effect in newborn mice. More recently, it was shown that co-administration of GM-CSF blocks the development of unresponsiveness in this model [59], adding a critical piece to the mechanistic puzzle. The simplest hypothesis is that GM-CSF-responsive cells such as DC are immature during the short neonatal period, leading to increased susceptibility to tolerance. The activity of neonatal APC may be partially ameliorated by increasing the content of unmethylated oligodeoxynucleotides [58], that results in more substantial adult-type IL-12 production and a shift to Th1 responses. Alternatively, co-administration of plasmids expressing Th1-driving cytokines (IL-12 or IFN-γ) was able to direct the neonatal immunity triggered by genetic vaccination, toward a Th1 profile [31]. These studies are in support of an earlier report that showed significant differences between T-cell cytokine profiles subsequent to neonatal versus adult vaccination with a plasmid expressing influenza hemagglutinin [61]. Whereas adult intramuscular immunization resulted in strongly biased Th1 response, the neonatal DNA vaccination led to a mixed, Th1/Th2 profile.

The differential responsiveness of neonates and adults to plasmid vaccines may create the opportunity to modulate immune responses using this strategy. Recently, we approached this issue by using plasmid vaccination with a

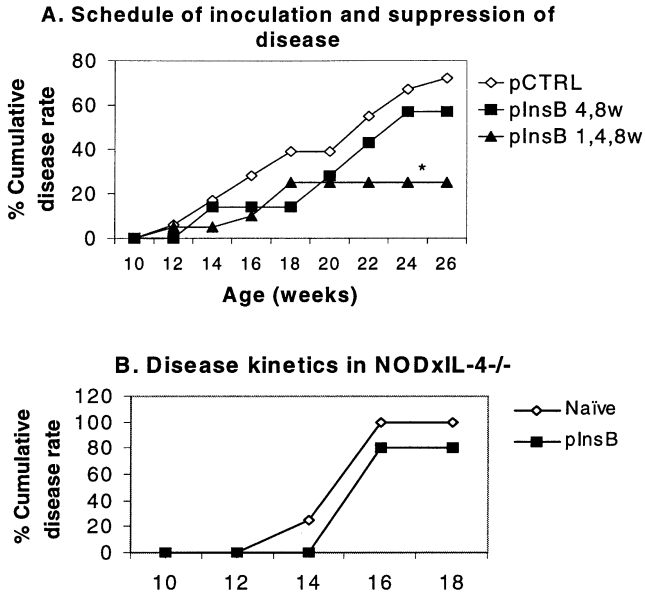

Fig. 2. (A) Female NOD mice were injected intramuscularly with plasmids expressing insulin B (pInsB) or control plasmid (pCTRL), at the age of 1, 4 and 8 weeks or only at 4 and 8 weeks (pInsB); * P of log-rank test < 0.05. (B) Alternatively, IL-4null NOD mice were injected using the same three-inoculation protocol, with insulin B-expressing plasmid. The results were expressed as % hyperglycemic animals.

self-antigen (insulin B chain) that has been previously shown to block autoimmune diabetes in an antigen-mimicry model [62]. Priming of newborn NOD mice with this plasmid, followed by two boosts (at 4 and 8 weeks) was followed by substantial suppression of disease (Fig. 2A). This was associated with Th2 shift in the spleen and expansion of both Th1 and Th2 arms in the pancreas-draining lymph nodes [37]. The specificity was demonstrated by the inability of control plasmid lacking the insulin gene, to influence the disease. The neonatal priming was critical, since the mice that were not primed during early life inexorably progressed to full-blown disease (Fig. 2A). This was not simply an issue of number of inoculations, since four administrations of plasmid failed to further increase the protective efficacy. Based on previous studies of neonatal DNA vaccination as well as T-cell profiles generated by plasmid vaccination with insulin B chain, we postulated that a T-cell shift is responsible for the protection against disease in the NOD model. This was confirmed by the lack of protection of NOD IL-4–/– mice subsequent to plasmid vaccination with the insulin B chain (Fig. 2B).

Together, these data demonstrate that by early plasmid vaccination, it is possible to generate a deviated/regulatory immune response that remains imprinted. In addition, IL-4 is a cytokine associated with T cells primed by neonatal DNA vaccines, that can be of value for the prophylaxis of autoimmune diseases.

4. Conclusions and directions

Pre-clinical studies during the last decade showed that neonatal DNA vaccination most often leads to induction of immunity rather than unresponsiveness, despite the immaturity of the immune repertoire and of APC. Immunostimulatory motifs associated with plasmid DNA are responsible for the activation of APC, resulting in induction of IL-12 dependent, IFN-γ-producing Th1 and cytotoxic cells. When this mechanism is not sufficient, additional APC-activating signals may be delivered (i.e. GM-CSF) for the purpose of optimizing the response to neonatal DNA vaccines. As a rule, neonatal DNA vaccines are substantially more effective in inducing T1 immunity than conventional vaccines.

Nevertheless, there are differences in the magnitude and quality of immune responses triggered by neonatal and adult DNA vaccination. In contrast to adults, the neonates display a higher propensity to develop Th2 immunity subsequent to DNA vaccination, which co-exists with the Th1 component. Secondly, due to the relative immaturity of the immune system, the magnitude of immune response generated by neonatal DNA vaccination is usually reduced compared to that triggered by adult immunization.

The potential advantages of neonatal vaccination with DNA-based expression vectors are (i) induction of broad humoral, Th and CTL responses; (ii) lack of side effects due to vector replication, associated with live virus immunization of infants; (iii) lack of interference of maternal antibodies with the T-cell responses elicited by neonatal DNA vaccines and (iv) the practical feasibility of designing and manipulating DNA vaccines.

There are still issues to be resolved before the DNA vaccines would be in a position to be seriously considered for neonatal vaccination of humans. First, more safety data are needed to rule out possible side effects like genome integration and oncogenesis, particularly taking into account that neonatal tissues comprise many proliferating cells. Secondly, there is a need for means to improve the efficiency in terms of the magnitude of immunity, relative to vaccine dose. This is particularly important, since to date, the results obtained in phase I/II clinical trials with prototype DNA vaccines administered to adult volunteers resulted in responses of rather limited magnitude [63]. However, since the immune system of newborn primates is more matured compared to that of rodents, the likelihood of high zone tolerance may be even more reduced in humans. Supporting this notion, it has been recently demonstrated that even fetal administration of DNA vaccine to non-human primates resulted in induction of immunity rather than tolerance [64].

One avenue to increase the immune response is by improving the in vivo transfection rate and/or antigen expression levels. Another way is to deliver the vaccine locally, for the purpose of generating mucosal immunity. Due to known quantitative differences between mucosal associated lymphoid tissue in rodents and primates, more

work is required to validate the observations obtained mainly in mice. Still, a different avenue consists in priming with DNA vaccines that induce adult-type immune memory, followed by boost with conventional vaccines, that readily expands this memory pool. Immunization with conventional vaccines formulated with CpG motifs, while retaining the benefits of DNA vaccines, may be superior regarding the magnitude of immune response and the safety profile.

Finally, neonatal DNA vaccines may be used to modulate emerging immune reactions against self-antigens, with implications for prophylaxis of autoimmune diseases in predisposed individuals. Since the outcome in terms of Th1/Th2 profile is controlled by the number of species-specific immunostimulatory motifs, a practical application for this concept awaits additional studies.

References

[1] C.A. Siegrist, Neonatal and early life vaccinology, Vaccine 19 (2001) 3331–3346.

[2] R. Billingham, L. Brent, P. Medawar, Quantitative studies on tissue transplantation immunity, III. Actively aquired tolerance, Proc. R. Soc. London 239 (1956) 44–46.

[3] J.P. Ridge, E.J. Fuchs, P. Matzinger, Neonatal tolerance revisited: turning on newborn T cells with dendritic cells, Science 271 (1996) 1723–1726.

[4] A.J. Yellen, W. Glenn, V.P. Sukhatme, X. Cao, J.G. Monroe, Signaling through surface IgM in tolerance-susceptible immature murine B lymphocytes. Developmentally regulated differences in transmembrane signaling in splenic B cells from adult and neonatal mice, J. Immunol. 146 (1991) 1446–1454.

[5] R.J. Wecksler, J.G. Monroe, Immature B lymphocytes are deficient in expression of the src-family kinases p59fyn and p55fgr1, J. Immunol. 154 (1995) 1919–1929.

[6] A. Durandy, G. De Saint Basile, G. Lisowska-Grospierre, G.F. Gauchat, M. Forveille, R.A. Kroczek, A. Bonnefoy, A. Fischer, Undetectable CD40 ligand expression on T cells and low B cell responses to CD40 binding agonists in human newborns, J. Immunol. 154 (1995) 1560–1568.

[7] B. Adkins, A. Ghanei, K. Hamilton, Developmental regulation of IL-4, IL-2, and IFN-gamma production by murine peripheral T lymphocytes, J. Immunol. 151 (1993) 6617–6626.

[8] B. Adkins, A. Ghanei, K.J. Hamilton, Up-regulation of murine neonatal T helper cell responses by accessory cell factors, J. Immunol. 153 (1994) 3378–3385.

[9] C.Y. Lu, E.G. Calamai, E.R. Unanue, A defect in the antigen-presenting function of macrophages from neonatal mice, Nature 282 (1979) 327–329.

[10] M. Kobayashi, H. Asano, Y. Fujita, T. Hoshino, Development of ATPase-positive, immature Langerhans cells in the fetal mouse epidermis and their maturation during the early postnatal period, Cell. Tissue Res. 248 (1987) 315–322.

[11] M. Carroll, The role of complement in B cell activation and tolerance, Adv. Immunol. 74 (2000) 61–88.

[12] B. Wolach, T. Dolfin, R. Regev, S. Gilboa, M. Schlesinger, The development of the complement system after 28 weeks gestation, Acta. Paediatr. 86 (1997) 523–527.

[13] T.M. Ross, Y. Xu, R. Bright, H.L. Robinson, C3d enhancement of antibodies to hemagglutinin accelerates protection against influenza virus challenge, Nat. Immunol. 1 (2000) 127–131.

[14] G.C. Liggins, The role of cortisol in preparing the fetus for birth, Reprod. Fertil. Dev. 6 (1994) 141–150.

[15] G. Mor, G. Yamshchikov, M. Sedegah, M. Takeno, R. Wang, R.A. Houghten, S. Hoffman, D.M. Klinman, Induction of neonatal tolerance by plasmid DNA vaccination of mice, J. Clin. Invest. 98 (1996) 2700–2705.

[16] A. Bot, S. Bot, A. Garcia-Sastre, C.A. Bona, DNA immunization of newborn mice with a plasmid-expressing nucleoprotein of influenza virus, Viral Immunol. 9 (1996) 207–210.

[17] M. Monteil, M.F. le Potier, J. Guillotin, R. Cariolet, C. Houdayer, M. Eloit, Genetic immunization of seronegative one-day-old piglets against pseudorabies induces neutralizing antibodies but not protection and is ineffective in piglets from immune dams, Vet. Res. 27 (1996) 443–452.

[18] Y. Wang, Z. Xiang, S. Pasquini, H. Ertl, Immune response to neonatal genetic immunization, Virology 228 (1997) 278–284.

[19] E. Manickan, Z. Yu, B.T. Rouse, DNA immunization of neonates induces immunity despite the presence of maternal antibody, J. Clin. Invest. 100 (1997) 2371–2375.

[20] X. Martinez, C. Brandt, F. Saddalah, C. Tougne, C. Barrios, F. Wild, G. Dougan, P.H. Lambert, C.A. Siegrist, DNA immunization circumvents deficient induction of T helper type 1 and cytotoxic T lymphocyte responses in neonates and during early life, Proc. Natl. Acad. Sci. USA 94 (1997) 8726–8731.

[21] C. Rollier, C. Charollois, C. Jamard, C. Trepo, L. Cova, Early life humoral response of ducks to DNA immunization against hepadnavirus large envelope protein, Vaccine 18 (2000) 3091–3096.

[22] M. Sarzotti, T.A. Dean, M.P. Remington, C.D. Ly, P.A. Furth, D.S. Robbins, Induction of cytotoxic T cell responses in newborn mice by DNA immunization, Vaccine 15 (1997) 795–797.

[23] X. Martinez, X. Li, J. Kovarik, M. Klein, P.H. Lambert, C.A. Siegrist, Combining DNA and protein vaccines for early life immunization against respiratory syncytial virus in mice, Eur. J. Immunol. 29 (1999) 3390–3400.

[24] D.E. Hassett, J. Zhang, J.L. Whitton, Neonatal DNA immunization with a plasmid encoding an internal viral protein is effective in the presence of maternal antibodies and protects against subsequent viral challenge, J. Virol. 71 (1997) 7881–7888.

[25] M.L. Bagarazzi, J.D. Boyer, M.A. Javadian, M. Chattergoon, K. Dang, G. Kim, J. Shah, B. Wang, D.B. Weiner, Safety and immunogenicity of intramuscular and intravaginal delivery of HIV-1 DNA constructs to infant chimpanzees, J. Med. Primatol. 26 (1997) 27–33.

[26] A.M. Prince, R. Whalen, B. Brotman, Successful nucleic acid based immunization of newborn chimpanzees against hepatitis B virus, Vaccine 15 (1997) 916–919.

[27] A. Bot, M. Shearer, S. Bot, C. Woods, J. Limmer, R. Kennedy, R. Casares, C. Bona, Induction of antibody response by DNA immunization of newborn baboons against influenza virus, Viral Immunol. 12 (1999) 91–96.

[28] A. Bot, M. Shearer, S. Bot, M. Avriette, A. Garcia-Sastre, G. White, C. Woods, R. Kennedy, C. Bona, Induction of immunological memory in baboons primed with DNA vaccine as neonates, Vaccine 19 (2001) 1960–1967.

[29] A. Bot, S. Antohi, S. Bot, A. Garcia-Sastre, C. Bona, Induction of humoral and cellular immunity against influenza virus by immunization of newborn mice with a plasmid bearing a hemagglutinin gene, Int. Immunol. 9 (1997) 1641–1650.

[30] T. Pertmer, H.L. Robinson, Studies on antibody responses following neonatal immunization with influenza hemagglutinin DNA or protein, Virology 257 (1999) 406–414.

[31] T. Pertmer, A.E. Oran, C.A. Mandorin, H.L. Robinson, Th1 genetic adjuvants modulate immune responses in neonates, Vaccine 19 (2001) 1764–1771.

[32] A. Bot, S. Bot, A. Garcia-Sastre, C. Bona, Protective cellular immunity against influenza virus induced by plasmid inoculation of newborn mice, Dev. Immunol. 5 (1998) 197–210.

[33] A. Bot, S. Bot, C.A. Bona, Enhanced protection against influenza virus of mice immunized as newborns with a mixture of plasmids expressing hemagglutinin and nucleoprotein, Vaccine 16 (1998) 1675–1682.

[34] C.L. Brazolot Millan, R. Weeratna, A.M. Krieg, C.A. Siegrist, H.L. Davis, CpG DNA can induce strong Th1 humoral and cell-mediated immune responses against hepatitis B surface antigen in young mice, Proc. Natl. Acad. Sci. USA 95 (1998) 15553–15558.

[35] D.E. Hassett, J. Zhang, M. Slifka, L.J. Whitton, Immune responses following neonatal DNA vaccination are long-lived, abundant, and qualitatively similar to those induced by conventional immunization, J. Virol 74 (2000) 2620–2627.

[36] J.M. Riberdy, A. Zirkel, S. Surman, J. Hurwitz, P.C. Doherty, Cutting edge: culture with high doses of viral peptide induces previously unprimed CD8(+) T cells to produce cytokine, J. Immunol. 167 (2001) 2437–2440.

[37] A. Bot, D. Smith, S. Bot, A. Hughes, T. Wolfe, L. Wang, C. Woods, M. von Herrath, Plasmid vaccination with insulin B chain prevents autoimmune diabetes in nonobese diabetic mice, J. Immunol. 167 (2001) 2950–2955.

[38] P. Albrecht, F.A. Ennis, E.J. Saltzman, S. Krugman, Persistence of maternal antibody in infants beyond 12 months: mechanism of measles vaccine failure, J. Pediatr. 91 (1977) 715–718.

[39] B.R. Murphy, R.A. Olmsted, P.L. Collins, R.M. Chanock, G.A. Prince, Passive transfer of respiratory syncytial virus (RSV) antiserum suppresses the immune response to the RSV fusion (F) and large (G) glycoproteins expressed by recombinant vaccinia viruses, J. Virol. 62 (1988) 3907–3910.

[40] C. Van Maanen, G. Bruin, E. deBoer-Liujtze, G. Smolders, G.F. de Boer, Interference of maternal antibodies with the immune response of foals after vaccination against equine influenza, Vet. Q. 14 (1992) 13–17.

[41] P.G. Harte, J.B. DeSouza, J.H.L. Playfair, Failure of malaria vaccination in mice born to immune mothers, Clin. Exp. Immunol. 49 (1982) 509–516.

[42] H.O. Aghomo, O.O. Oduye, C.E. Ruprecht, The serological response of young dogs to the Flury LEP strain of rabies virus vaccine, Vet. Res. Commun. 14 (1990) 415–425.

[43] A. Bot, S. Bot, S. Antohi, K. Karjalainen, C. Bona, Kinetics of generation and persistence on membrane class II molecules of a viral peptide expressed on foreign and self proteins, J. Immunol. 157 (1996) 3436–3442.

[44] K.L. Legge, B. Min, J.J. Bell, J.C. Caprio, L. Li, R.K. Gregg, H. Zaghouani, Coupling of peripheral tolerance to endogenous interleukin 10 promotes effective modulation of myelin-activated T cells and ameliorates experimental allergic encephalomyelitis, J. Exp. Med. 191 (2000) 2039–2052.

[45] K.W. Moore, R. de Waal Malefyt, R.L. Coffman, A. O'Garra, Interleukin-10 and the interleukin-10 receptor, Annu. Rev. Immunol. 19 (2001) 683–765.

[46] A. Regnault, D. Lankar, V. Lacabanne, A. Rodriguez, C. Thery, M. Rescigno, T. Saito, S. Veerbeek, C. Bonnerot, P. Ricciardi-Castagnolli, Fcgamma receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization, J. Exp. Med. 189 (1999) 371–380.

[47] C.A. Siegrist, C. Barrios, X. Martinez, C. Brandt, M. Berney, M. Cordova, J. Kovarik, P.H. Lambert, Influence of maternal antibodies on vaccine responses: inhibition of antibody but not T cell responses allows successful early prime-boost strategies in mice, Eur. J. Immunol. 28 (1998) 4138–4148.

[48] C.A. Siegrist, M. Cardova, C. Brandt, C. Barrios, M. Berney, C. Tougne, J. Kovarik, P.H. Lambert, Determinants of infant responses to vaccines in presence of maternal antibodies, Vaccine 16 (1998) 1409–1414.

[49] T. Pertmer, A.E. Oran, J.M. Moser, C.A. Madorin, H.L. Robinson, DNA vaccines for influenza virus: differential effects of maternal antibody on immune responses to hemagglutinin and nucleoprotein, J. Virol. 74 (2000) 7787–7793.

[50] D.L. Radu, S. Antohi, A. Bot, A. Miller, A. Mirarchi, C. Bona, Effect of maternal antibodies on influenza virus-specific immune response elicited by inactivated virus and naked DNA, Scand. J. Immunol. 53 (2001) 475–482.

[51] P.J. Lewis, van Drunen Little-Van Den Hurk, L.A. Babiuk, Induction of immune responses to bovine herpesvirus type 1 gD in passively immune mice after immunization with a DNA-based vaccine, J. Gen. Virol. 80 (1999) 2829–2837.

[52] P.D. Simitsek, D.G. Campbell, A. Lanzavecchia, N. Fairweather, C.J. Watts, Modulation of antigen processing by bound antibodies can boost or suppress class II major histocompatibility complex presentation of different T cell determinants, Exp. Med. 181 (1995) 1957–1965.

[53] K.J. Liu, V.S. Parkih, P.W. Tucker, B.S. Kim, Surface immunoglobulins mediate efficient transport of antigen to lysosomal compartments resulting in enhanced specific antigen presentation by B cells, Eur. J. Immunol. 24 (1994) 2755–2763.

[54] S. Casares, K. Inaba, T.D. Brumeanu, R. Steinman, C.A. Bona, Antigen presentation by dendritic cells after immunization with DNA encoding a major histocompatibility complex class II-restricted viral epitope, J. Exp. Med. 186 (1997) 1481–1486.

[55] O. Akbari, N. Panjwani, S. Garcia, R. Tascon, D. Lowrie, B. Stockinger, The HSC73 molecular chaperone: involvement in MHC class II antigen presentation, J. Exp. Med. 189 (1999) 169–178.

[56] R.D. Weeratna, C.L. Brazolot Millan, M.J. McCluskie, C. Siegrist, H. Davis, CpG, ODN can re-direct the Th bias of established Th2 immune responses in adult and young mice, FEMS Immunol. Med. Microbiol. 30 (2001) 241–247.

[57] P. Seiler, M.A. Brundler, C. Zimmerman, D. Weibel, M. Bruns, P. Hengartner, R.M. Zinkernagel, Induction of protective cytotoxic T cell responses in the presence of high titers of virus-neutralizing antibodies: implications for passive and active immunization, J. Exp. Med. 187 (1998) 649–654.

[58] J. Kovarik, P. Bozzotti, L. Loveman-Homan, M. Pihlgren, H.L. Davis, P.H. Lambert, A.M. Krieg, C.A. Siegrist, CpG oligodeoxynucleotides can circumvent the Th2 polarization of neonatal responses to vaccines but may fail to fully redirect Th2 responses established by neonatal priming, J. Immunol. 162 (1999) 1611–1617.

[59] K.J. Ishii, W.R. Weiss, D.M. Klinman, Prevention of neonatal tolerance by a plasmid encoding granulocyte-macrophage colony stimulating factor, Vaccine 18 (1999) 703–710.

[60] M. Ichino, G. Mor, J. Conover, W.R. Weiss, M. Takeno, K.J. Ishii, D.M. Klinman, Factors associated with the development of neonatal tolerance after the administration of a plasmid DNA vaccine, J. Immunol. 162 (1999) 3814–3818.

[61] A. Bot, S. Antohi, C. Bona, Immune response of neonates elicited by somatic transgene vaccination with naked DNA, Front. Biosc. 2 (1997) 173–188.

[62] B. Coon, L.L. An, M.G. Whitton, M.G. von Herrath, DNA immunization to prevent autoimmune diabetes, J. Clin. Invest 104 (1999) 189–194.

[63] R. Wang, J. Epstein, F.M. Baraceros, E.J. Charoenvit, D.J. Carucci, R.C. Hedstrom, N. Rahardjo, T. Gay, P. Hobart, R. Stout, T.R. Jones, T.L. Richie, S.E. Parker, D.L. Doolan, J. Norman, S.L. Hoffman, Induction of CD4(+) T cell-dependent CD8(+) type 1 responses in humans by a malaria DNA vaccine, Proc. Natl. Acad. Sci. USA 98 (2001) 10817–10822.

[64] A.M. Watts, J.R. Stanley, M.H. Shearer, P.S. Hefty, R.C. Kennedy, Fetal immunization of baboons induces a fetal-specific antibody response, Nat. Med. 5 (1999) 427–430.

[65] S. Van Drunen Little-van Den Hurk, R.P. Braun, P.J. Lewis, B.C. Karvonen, L.A. Babiuk, P.J. Griebel, Immunization of neonates with DNA encoding a bovine herpesvirus glycoprotein is effective in the presence of maternal antibodies, Viral Immunol. 12 (1999) 67–77.