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A single immunization near birth elicits immediate and lifelong protective immunity

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ABSTRACT

Most existing vaccines do not induce protective immunity immediately following birth, nor do they retain protective efficacy in the latter years of life without booster doses. Using a mouse model, we present evidence that a live-replicating vaccine administered only once shortly after birth was able to induce both immediate and lifelong protection. Newborn mice immunized with a safe, highly attenuated strain of *Listeria monocytogenes* (Lm) were already protected by day 7 post-vaccination when challenged with a virulent strain of Lm. Furthermore, all mice remained fully protected for 2 years after only a single immunization. Vaccine-specific T cell immune responses were still detectable 2 years later, indicating long-lived immune memory even in neonatal vaccine recipients. Analysis of memory precursor subsets, specific for antigens homologous to Lm or a model vaccine (Ova), demonstrated remarkable similarity between adult and neonatal vaccine recipient effector and central memory CD8 T cell development. The magnitude of expansion of antigen specific memory T cells post-infectious challenge correlated with protection in both groups. This is the first direct evidence that vaccination—even in the absence of a booster dose—is capable of inducing immediate and lifelong protective immune memory regardless of age at the time of initial vaccination.

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1. Introduction

Vaccination is rightly hailed as one of the greatest medical interventions, but for many vaccine-preventable diseases we remain incapable of protecting the most vulnerable—our youngest and our oldest [1,2]. The infant's immune response to routinely administered vaccines is often suboptimal and frequently requires boosting to reach protective levels that are readily achieved in older children or adults [3]. Infants also appear to be more susceptible to infections [4,5], prompting the WHO to recommend an earlier vaccination schedule in an attempt to curb infant mortality in areas where the risk of infection is highest [6]. Vaccination at birth would indeed offer operational savings, and result in greater vaccine coverage [7,8]. However, the development of safe and effective neonatal vaccines is hampered by a limited understanding of ways to optimally induce early and long-lasting protective immune responses in the context of the neonatal immune system [9]. Live-replicating vaccines are expected to produce more robust and long lived immunity irrespective of when in life they are administered, but most childhood immunization utilizes inactivated or subunit vaccines [10]. The only 2 live vaccines currently used in neonates are BCG and OPV, which either lack life-long protective efficacy [11] or require booster vaccination [12]. An optimal vaccine would induce protective immunity within the neonatal immunological context.

When compared to adults, neonates appear to have a reduced capacity to produce T-helper type 1 (Th1) cytokines that support cell-mediated immunity [13], and also seem to be less capable of inducing and maintaining long-term Th1-CD4 and CD8 T cell immune memory [14]. Neonates instead appear to favor T-helper type 2 (Th2) or tolerogenic responses [15–17]. Similar to the reduced protective immune function in early life, the immune system of the elderly also appears to lean towards a tolerogenic phenotype with an increasing abundance of suppressor cells [18], and with poorly functioning antigen-presenting cells (APCs) and T cells [2]. Collectively, these ontogenic phenomena have contributed to the commonly held belief that the newborn and the elderly develop and/or maintain immunologic memory poorly, that vaccines given during this time are less effective, and that long-lasting

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protection in these disparate age groups requires repeated booster immunization. Indeed, this appears to hold true with most commonly used vaccines [2,5,19–21].

We show here, with only a single immunization with attenuated Lm administered to both newborn and adult mice, that fully protective immunity from challenge with a vaccine homologous pathogen was induced immediately and then lasted a lifetime. Development of CD8 T cell immune memory subtypes was nearly identical between neonatal and adult vaccine recipients, and upon secondary challenge the magnitude of IFN-γ^+ T cell expansion correlated with protection in both groups. However, specific measures of correlates of protection (CoP) were more readily identifiable in mice immunized as adults. While a previous report from our group demonstrated generation of immune memory responses in both neonatal and adult vaccine recipients [9], we establish here the lifelong persistence of protective immunological memory responses in both groups. This represents the first indication that lifelong protective immune memory is achievable through vaccination of neonates, without the need for booster doses.

2. Materials and methods

2.1. Mice

Murine pups were vaccinated at day 6 of life, which is the age these mice are closest to human neonates with respect to the maturational status of the immune system [1]. Adult mice were 6 weeks of age at the time of vaccination. The mice were F_1 mice expressing both H-2^b and H-2^d MHC alleles (C57BL/6 × BALB/c). In pilot experiments we found that the F_1 mouse (H-2^b × H-2^d) immune responses to stimulation with the $Ova_{257\text{-}264}$ and $LLO_{189\text{-}201}$ peptides (both presented by the H-2^b haplotype [22–24]) were indistinguishable from C57BL/6 mouse responses (only H-2^b) (data not shown). While the current set of experiments focused only on these 2 H-2^b restricted epitopes, we chose to continue using F1 mice expressing both haplotypes to allow direct comparison of our current data to our previous findings on neonatal immunization strategies using Lm. After vaccination, pups were housed with the unvaccinated mother and weaned at 21 days of age. Animals were housed under specific pathogen-free conditions at the Child and Family Research Institute of the University of British Columbia. The Institutional Animal Care and Use Committee approved animal use protocols.

2.2. Bacterial strains, immunization and infectious challenge

Lm Δ*actA* strain DPL1942, and Lm-Ova was kindly provided by Drs. N. Freitag and D. Portnoy (University of Illinois-Chicago and University of California-Berkley, respectively), and Dr. H. Shen (University of Pennsylvania, Philadelphia, PA), respectively. Lm Δ*actA*-Ova (attLm-Ova), which lacks the *actA* virulence gene but expresses the heterologous model vaccine peptide ovalbumin (Ova), was provided by Dr. S.S. Way (University of Minnesota) [25]. This strain was used for adult and neonatal vaccinations. All strains were grown to late-log phase (OD_{600} = 1.0) at 37 °C, washed in normal saline, and stored frozen at −80 °C in normal saline solution containing 20% glycerol. Frozen stocks were diluted to 100 μl in normal saline and injected i.p. for both immunization and challenge. Live Lm in lysates of infected spleens and livers were determined near the peak of bacterial burden (4 days post-infection) as described [25], by homogenizing organ tissue and plating serial dilutions on Lm growth agar (brain-heart infusion). Lower limit of detection was 30 and 50 colony-forming units (CFU) per organ for spleen and liver, respectively. We determined the minimal challenge dose of Lm expressing Ova (Lm-Ova), that was necessary to induce signs of moribundity (piloerect fur, hunched position, hypoactive, weight loss) in 100% of recipients in each age group. These doses were 1×10^4, 1×10^7, and 5×10^5 for 12-day (1 week post neonatal vaccination), 4-month and 2-year old mice, respectively. All immunizations were at a dose of 1×10^4 attLm-Ova.

2.3. Enumeration of antigen-specific T cells

Intracellular cytokine (ICC) staining was performed as described [25] using reagents from eBioscience (eBio) (San Diego, CA) and BD Pharmingen (BD) (Mountain View, CA). Splenocyte suspensions were prepared by homogenizing spleens between two sterile glass slides, subjected to RBC lysis and then filtered through a 70-μm mesh and washed in RPMI 1640 media. Splenocytes were incubated for five hours in 200 μl of RPMI 1640 medium supplemented with 10% FCS (Hyclone, Logan, UT), L-glutamine, penicillin, and streptomycin in the presence of the indicated $Ova_{257\text{-}264}$ or $LLO_{189\text{-}201}$ peptide (10^{-6} M) and Brefeldin A. Cells were subsequently permeabilized with Cytoperm solution (BD), and stained with APC-Cy7-labeled anti-CD3 (BD), either PerCP-labeled anti-CD8 or anti-CD4 (BD), and PE-labeled anti-IFN-γ (eBio) for 45 min at room temperature and fixed in a 1% paraformaldehyde in PBS solution. Antigen-specific surface stains were performed on non-permeabilized cells using APC-Cy7-labeled anti-CD3 (BD), PerCP-labeled anti-CD8 (BD), APC-labeled anti-KLRG1 (eBio), PeCy7-labeled anti-CD127 (eBio), FITC-labeled anti-CD43 (eBio), Pacific Blue-labeled anti-CD62L (eBio), and PE-labeled Ova-loaded MHC class I tetramer. Stained cells were acquired uncompensated on a FACSAria flow cytometer (BD), and analyzed using FlowJo software (TreeStar, Ashland, OR). This publication complies with the minimal information about a flow cytometry experiment (MIFlowCyt) standard for reporting as outlined by Lee et al. [26]; detailed additional information as required by the MIFlowCyt standard is provided in Appendix 1.

2.4. Statistics

Percent protection was calculated as percent reduction, derived from the ratio of CFUs isolated from controls to CFUs isolated from the experimental groups (% protection = 1 − [CFU experimental/CFU control] × 100; max = 100%). The differences in geometric mean CFUs, and the differences in the percentages of activated $CD3^+$ splenocytes were evaluated using the Student's *t*-test, with $p < 0.05$ taken as statistically significant. Correlation of protection analysis between protection and cytokine production was assessed per mouse by using Prism (GraphPad Software Inc.) to conduct a two-tailed Pearson correlation analysis; $p < 0.05$ was taken as statistically significant.

3. Results

3.1. Immunization with a live-replicating vaccine protects immediately and for life regardless of age at vaccination

To date there have been no reports of both immediate and lifelong protection elicited from a single neonatal vaccination in either humans or animal models. Furthermore, most studies of neonatal vaccination have only shown substandard or short-lived protective immune responses after early life vaccination [3,5,14]. Based on our previous findings demonstrating robust recall immune responses in adult mice immunized with Lm as newborns [9,27,28], we hypothesized that a single immunization given around birth would induce protection during the neonatal period and that this protection would persist for the animal's lifespan without requiring any booster doses. We therefore set out to determine if a single neonatal

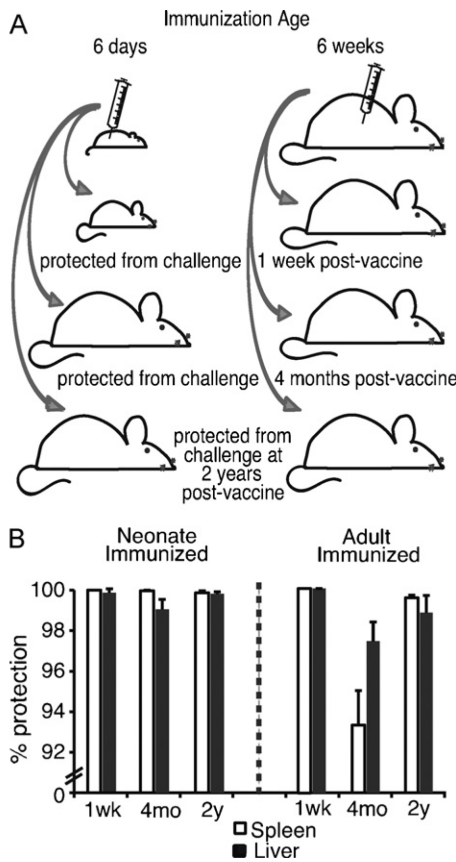


Fig. 1. Mice immunized during the neonatal period were protected for life. Mice were immunized with 1×10^4 attLm-Ova on day 6 (Neonate) or week 6 of life (Adult). Challenge doses of virulent Lm-Ova induced moribundity in all age-matched naïve controls. 1×10^4, 1×10^7 and 5×10^5 were administered to 12 day (7 days post neonatal vaccination), 4 month and 2 year old mice, respectively. (A) There was 100% moribund-free survival in immunized mice; all naive mice developed signs of moribundity by 4 days post-challenge. (B) Shown is the percent reduction in CFU in the infected, but immunized, group from the CFU detected in the infected unimmunized age-matched naïve controls. 100% protection = undetectable CFU/organ; 0% = no decrease in CFU/organ relative to naive controls. All immunized groups at each time point were significantly protected when compared to naive controls ($p < 0.05$); e.g. 4414 ± 3423/5800 ± 3242 CFU (mean ± SEM) isolated from spleens (white bar)/livers (black bar) of challenged mice at 2-years post neonatal vaccination; heavy infectious burdens of 3,257,000 ± 1,463,750/3,325,067 ± 529,369 CFU were isolated from the spleens and livers of age-matched infected, but previously naïve, control mice. Bar, SEM; n = 6–9 per group.

immunization with Lm could provide immediate protection early in life, and how long such protection would last. To this end, we immunized 6-day old mice with attLm-Ova. Protective immunity in immunized mice was qualitatively assessed after infection with a virulent Lm strain administered at a dose sufficient to induce moribundity in 100% of naïve recipients. Protection was also quantified by enumerating the bacterial load (CFUs) in the spleen and the liver of immunized mice, and by comparing these to the CFUs isolated from age-matched naïve control (previously unimmunized) mice. Six to nine individual mice in each group were immunized as neonates (6 days old) or as adults (6 weeks old) and then challenged 7 days, 4 months, or 2 years later. The experimental setup is illustrated in Fig. 1A. Both neonatally- and adult-immunized mice were completely protected from infectious challenge as early as 1 week post-vaccination. No immunized mouse developed signs of moribundity, while all naïve control mice succumbed to infection within 4 days of challenge—i.e., vaccine-induced protection from moribund outcome resulting from virulent Lm-Ova infection equaled 100%. Quantitative measures of protection did not wane at any time point (7 days, 4 months, 2 years) post-immunization, with >90% reduction in CFU for each experimental group (% reduction of CFU/organ compared to infected age-matched naïve mice (Fig. 1B)). Importantly, this level of protection at day 4 post-challenge corresponds to sterilizing protection (i.e., undetectable CFU) by day 7 post-challenge [29,30]. Long-term immunity was demonstrated by significant reductions in bacterial load after challenge at 4 months post-vaccination (confirming our previous finding [9]). We were able to now extend this finding until the very end of the normal 2-year lifespan of a mouse (Fig. 1B). Specifically, 4 days after infectious challenge, we detected 4414 ± 3423 (mean ± standard error (SE)) CFU in the spleens and 5800 ± 3242 CFU in the livers of 2-year old mice that had been immunized as neonates, and 15,288 ± 4660 and 38,840 ± 27,954 in the spleens and livers, respectively, of 2-year old mice immunized as adults. This clearly shows that both groups were significantly protected ($p < 0.05$) relative to the CFU recovered from the spleens (3,257,000 ± 1,463,750) and livers (3,325,067 ± 529,369) of age-matched, infected but naïve control mice.

3.2. Memory cell subtypes (Tcm and Tem) develop at a similar rate and ratio irrespective of age of vaccination

Immune responses protecting from virulent Lm challenge have been shown to depend on the type of $CD8^+$ T cell immune memory that is developed after Lm-based vaccination: in a study employing adoptive transfer of TCR-transgenic T cells, it was demonstrated that effector memory T cell (Tem) responses were more protective against Lm than central memory T cells (Tcm) [31]. We set out to determine the development of these two immune memory subsets over the course of both the adult and neonatal primary response to vaccination. To this end, a memory development time-course experiment was performed, in which both neonatal and adult mouse splenocytes were stained for vaccine-specific memory precursor T cells ($CD3^+/CD8^+/CD43^+/OVA_{257-264}$ tetramer$^+$/$CD127^{hi}$/$KLRG1^{lo}$); CD43 is high in activated, expanding and migrating T cells [32], whereas $CD127^{hi}$ and $KLRG1^{lo}$ are commonly used markers for activated 1° memory precursor cells [33]; central memory T cells can be identified by surface expression of the CD62-ligand (CD62L) (Fig. 2A). Our data indicated that over the first month post-vaccination a similar percentage of vaccine-specific $CD8^+$ T cell memory precursor subsets develop in both age groups (Fig. 2B), with $CD8^+$ Tcm percentages ranging between 30% and 40% in adults as well as neonatal mice (Fig. 2C). Furthermore, we found very little fluctuation in the Tcm:Tem ratio from day 7 to day 21 post-vaccination. In our wild type mouse model (i.e., not involving TCR-transgenic T cells) $CD8^+$ T cells specific for Ova-antigen were too low in number by one month post-vaccination to allow detection above background Ova-specific Tem and Tcm cellular phenotypes.

3.3. Immune memory responses last a lifetime after only a single immunization of either newborn or adult mice

For commonly used vaccines, which are primarily inactivated or subunit vaccines, newborns require multiple booster doses to reach equivalent immune memory responses as similarly vaccinated children or adults [4,21]. Much like the neonatal period, long-term memory responses appear to also be poorly maintained in the elderly [2]. However, live vaccines are expected to elicit more optimal immune mediated protection irrespective of the age of vaccination [34]. We therefore set out to assess immune responses at the extreme ends of the age spectrum using live-attenuated Lm in our animal model (6 days of life and 2 years). To assess the longevity of Th1 immune memory responses in mice immunized with attLm-Ova, IFN-γ production was measured in antigen-specific CD8 and CD4 T cells 4 days post-challenge with virulent Lm-Ova. Immune

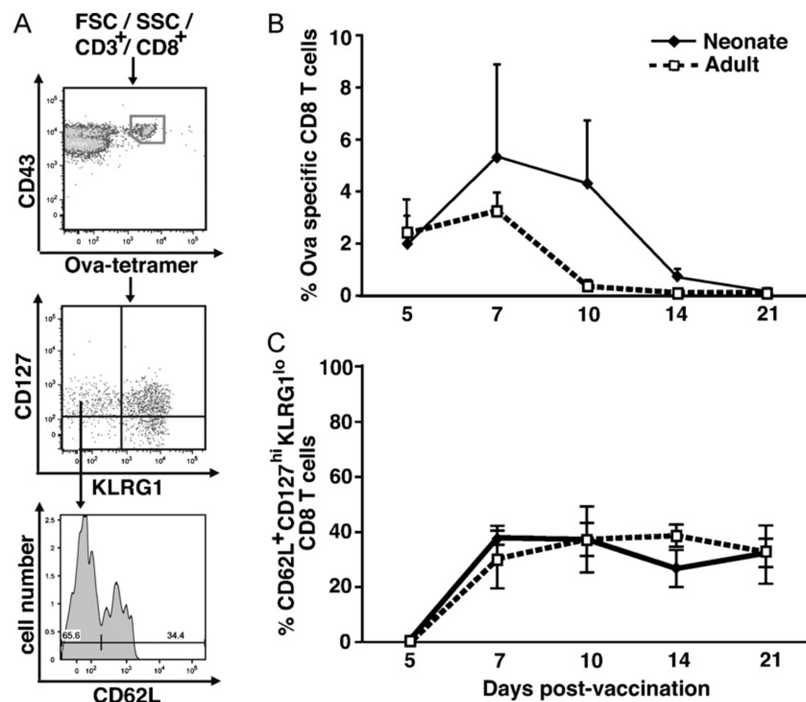


Fig. 2. Memory development occurred with similar kinetics and phenotype between adult and neonatal vaccine recipients. Mice were vaccinated with 1×10^4 of attLm-Ova, and Ova-specific T cells were detected in splenocytes by flow cytometry. (A) Identification of Ova-specific precursor memory cell subsets from total splenocytes. (B) Peak expansion of Ova-specific CD8 T cells in both neonates (solid line) and adults (dotted line) occurred by day 7 post-vaccine, but newborn vaccine recipient CD8 T cell contraction did not occur before day 10, while adult T cells began to contract after day 7. (C) Central memory CD8 T cells (Tcm) were developed already by day 7 in both newborn and adult vaccine recipients and proportion of Tcm were maintained equally over the course of the primary immune response for both groups. Bar, SEM; $n = 6$–9 per group.

memory was assessed at 4 months post-vaccination, as previously described [9], and compared to recall responses at 2 years post-vaccination. Recall responses were not assessed at the earlier time points, i.e., at 7 days post-immunization, as this time point corresponds to the near-peak primary immune response following immunization [9], thus precluding differentiation of primary vs. recall memory responses based on IFN-γ production. Neonatally and adult immunized mice were compared for responses to the MHC-I-restricted peptide $Ova_{257–264}$ (Fig. 3A) and the MHC-II-restricted peptide $LLO_{189–201}$ (Fig. 3B). Qualitative levels of cytokine production, on a per/cell basis, was similar for all groups at each time point, indicated by a similar mean fluorescent intensity (MFI) for IFN-γ (not shown). Both peptides used to measure antigen-specific cell responses represent the dominant CD8 ($Ova_{257–264}$) and CD4 ($LLO_{189–201}$) antigens, as determined in our previous study [9]. Significant levels of Ova-responsive (IFN-γ^+) CD8 T cells were detected after challenge at both 4 month and 2 years post-vaccination; however, at the earlier time point, vaccinated adults did not exhibit as strong a response as neonatally immunized mice. Both groups responded similarly when challenged at 2 years post-immunization (Fig. 3A). However, CD4 T cells from mice immunized as adults no longer responded significantly to LLO at 2 years post-immunization (Fig. 3B), while CD4 T cells of mice immunized as neonates maintained this capacity for up to 2 years.

3.4. Vaccine-specific IFN-γ+ T cells correlated significantly with protection from infectious challenge in both neonatally and adult immunized mice

A strong functional CD8 T cell IFN-γ^+ recall response has been shown to be associated with protection from virulent Lm [35]; however, evidence to support the validity of using markers such as cytokines for protective cell-mediated immunity is limited [36–38]. We therefore set out to determine the statistical relationship between the frequency of vaccine-specific T cells producing IFN-γ in response to pathogen-associated peptides, and the mouse's ability to clear an infectious challenge. A two-tailed Pearson correlation analysis demonstrated that the frequency of Ova-specific CD8 IFN-γ^+ responder T cells significantly correlated with clearance of Lm from the spleen in both neonatally and adult immunized mice, but clearance of Lm from the liver correlated with CD8 IFN-γ^+ T cell responses only in mice immunized as adults. Although the Pearson correlation coefficient (R-value) was lower for Ova (peptide to which attLm-Ova induced primary and memory responses are the strongest [9]) in the neonatally vaccinated group, the relationship still remained significant. Notably, neither group displayed a significant correlation between IFN-γ^+ CD4 T cell response and protection in the spleen; however, IFN-γ^+ CD4 T cell responses correlated significantly with protection in the liver, but only for mice immunized as neonates (Fig. 4).

4. Discussion

There is an urgent need to develop successful strategies to elicit lifelong immunity early in life with only a single immunization. Neonatal vaccination strategies promise to pay large dividends by protecting this most vulnerable group [6,39], by possibly increasing vaccine coverage [1,3], and would thus contribute to decreasing overall public health expenses [8]. However, vaccination in early life also poses challenges to vaccine development due to the rapidly changing status of the neonatal immune system [1,13,14,40]. The first step towards reaching this goal thus lies in identifying a system that successfully stimulates the neonate's unique immune system to generate long-term protective immune memory [41].

Lm is a potent activator of antigen-presenting cells (APCs) [42,43] and is currently under investigation for use as a vaccine vehicle in human adults [44–46]. Work from our group indicates that Lm elicits protective immune responses and can be safely used in murine newborns [9,28]. In the work presented here, we

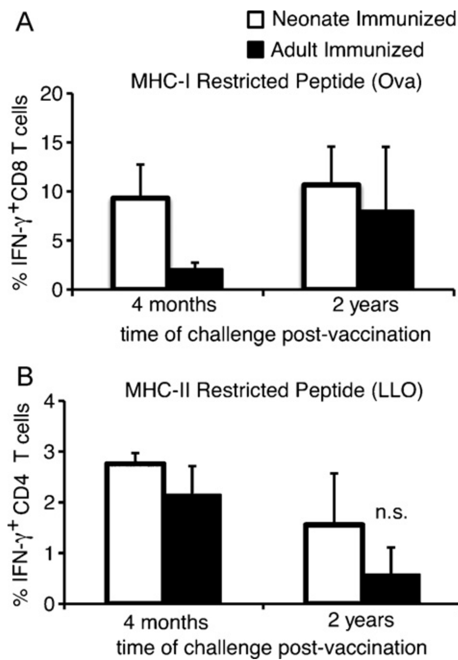
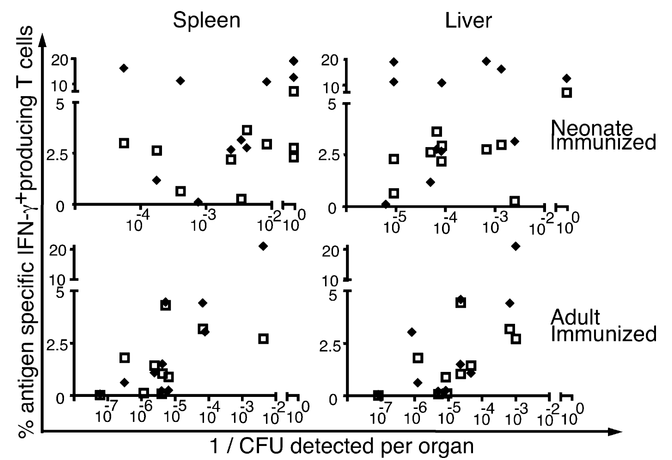


Fig. 3. Mice immunized during the neonatal and adult period developed memory T cells that lasted a lifetime. Mice immunized with attLm-Ova on day 6 of life or at 6 weeks of age, and non-immunized (naive) mice were infected with virulent Lm-Ova either 4 months or 2 years post-vaccination; cytokine production was assessed 4 days later by flow cytometric detection of IFN-γ+ CD8 and CD4 T cells after stimulation with MHC I restricted Ova peptide, or MHC II restricted LLO peptide, respectively. Percentages of IFN-γ+ T cells in naive controls were negligible and no-peptide control responses were subtracted from peptide stimulated responses for each mouse. Mean percent vaccine-peptide responsive IFN-γ+ CD8 and CD4 T cells are shown for neonatally immunized mice (white bars) and adult immunized mice (black bars). Responses from neonatally and adult immunized mice were significantly greater than naive-infected controls for both time points, except the adult CD4 T cell response, which was only detected as significant at 4 months. $p < 0.05$; n.s., not significant; bar, SEM; $n = 6$–9 per group.



* = correlation significance # = R-value	Neonate Immunized Spleen	Neonate Immunized Liver	Adult Immunized Spleen	Adult Immunized Liver
◆ IFN-γ+ Ova-specific CD8 T cells	* 0.49	n.s. 0.03	*** 0.93	*** 0.77
□ IFN-γ+ LLO-specific CD4 T cells	n.s. 0.27	** 0.61	n.s. 0.08	n.s. 0.24

* = p < 0.05 ** = p < 0.05 *** = p < 0.05

Fig. 4. Vaccine recall response of CD8 T cells correlated with protection from challenge with virulent Lm-Ova. Pearson correlation analysis was calculated on a per-mouse basis with clearance of infection from the spleen and liver. Shown is 1/CFU detected per organ (log scale) 4 days after infection of immunized mice, and % IFN-γ+ CD8 T cells (diamonds) or IFN-γ+ CD4 T cells (squares) on the *y*-axis. Statistical output for the Pearson correlation analysis is indicated for each peptide-specific cell measured. The numerical *R*-value indicates the strength of correlation. Student's *t*-test was performed to determine significance of correlation between the amplitude of antigen specific T cell expansion and the degree of protection (inverse of burden of infection) at day 4 post-challenge. $n = 12$ per group.

found that a single vaccination with attenuated Lm administered in the first week of life, induced protection from lethal challenge as early as 1 week post-vaccination. And this neonatally induced protection persisted for 2 years, the lifetime of a mouse, without the need for administering additional booster doses. Immune memory responses also lasted for the lifetime of the mouse and vaccine-specific T cells significantly correlated with protection from infectious challenge.

Challenges to effective vaccination of newborns appear to be a lack of strong antibody responses particularly to T cell independent antigens [19,21], a Th2 bias early in life [14,47], and suboptimal induction of long-lived immune memory [5]—all of which appear to necessitate the administration of booster doses later in life [1,13,15]. Live-attenuated-replicating vaccines have been shown to confer protective immunity even if given to neonates [12]. Our group has previously demonstrated that a live Lm-based vaccine administered around birth induces robust antigen-specific Th1 immune memory responses when challenged as adults [9,28]. But the primary goal of neonatal immunization has to be protection of the very young [48]. We thus set out to determine if an Lm-based neonatal immunization would induce immediate protection already in early life. We found that neonatal and adult mice immunized with attenuated Lm-Ova were fully protected 1 week following immunization (Fig. 1). This was the earliest time point that our system would allow us to determine sterilizing protection because the Lm vaccine inoculum takes up to 7 days to be cleared in mice immunized as neonates or as adults ([29,49] and data not shown). Our finding of early protection provides further support for the potential use of Lm as a vaccine vehicle for adults [44,46]. With respect to neonatal vaccine induced protection, other groups employing vaccine strategies not relying on Lm have shown protection following immunization in early life; however, protection in these model systems was apparent only 3 weeks to 1 month post-immunization [50–52], or only after administration of additional booster doses [48]. In light of these findings, our data indicating early life protective responses after a single neonatal vaccination support the notion that the neonatal immune system is not in principle unable to provide immediate protective responses, but is rather context dependent in that such responses rely on appropriate stimulation. Lm is a strong non-specific activator of the immune system and it will be of interest to decipher in great detail if the early protection we observed is in fact due to non-specific activation of the innate or adaptive immune system, or whether protection at the early time points post-immunization is already mediated by antigen specific adaptive mechanisms.

We further aimed to investigate the durability of protection, as protection of the very old is as important, and apparently as difficult to achieve as protection of the very young [19,53,54]. We followed mice immunized either near birth or as adults for the remainder of their natural life (~2 years), and were surprised to find that both neonatally and adult immunized mice were fully protected from lethal challenge at all time points analyzed—without ever receiving a booster dose (Fig. 1). To our knowledge, the durability of our Lm-based approach to vaccination, i.e., protection from birth onwards for life after only one immunization, is unparalleled and strongly argues for further exploration of Lm-based approaches for neonatal vaccination in humans.

As compared to inactivated or subunit vaccines, live-replicating vaccines are expected to induce more robust and long-lived immunity irrespective of when in life they are administered. However, outside of BCG, the only other live vaccine given to neonates is OPV. While BCG protects from disseminated TB early in life without the need for a booster, it does not protect from TB for life [11]. And attenuated live oral polio vaccine does not protect from wild-type polio challenge after one dose, irrespective of when in life it is given [55]. An Lm based neonatal vaccine platform holds promise to perform better than either of these 2 live neonatal vaccines. However, so far our model has only shown protection from homologous challenge, i.e., a highly attenuated Lm based vaccine can induce protection from a highly virulent wild type Lm challenge. While the finding that an attenuated Lm is a safe vaccine that can be used in neonatal mice and induce protective immunity for life is promising, it does not address the urgent need for protection from other more wide spread and clinically relevant infectious challenges. We are currently in the process of evaluating the possibility of Lm expressing heterologous antigens inducing protection from heterologous infections such as pertussis or malaria.

A recent report elucidated Tem as the most protective subtype of memory CD8 T cells in response to Lm challenge [31], indicating that the ratio of Tem:Tcm might be an important factor in predicting efficacy of vaccination [56]. Further, in humans Tem have been reported to dominate protective responses in short term (months–years) immunity, with Tcm gradually taking over for longer term (years–decades) immunity [57]. This phenomenon is likely due, in part, to less efficient homeostatic proliferation in Tem, compared to Tcm, resulting in depletion of Tem overtime. However, despite the greater protective efficacy of Tem responses to certain pathogens, the percent of Tcm may represent a positive predictor for the long-term protective capacity of an immune memory response [58,59].

Controversy surrounds the categorization of memory T cell subsets [60,61]. We chose to assess Tcm and Tem cells as these defined subsets have been best delineated for their protective efficacy in an Lm-based-vaccine model similar to our own (but with adult mice) [31]. We predicted that due to the apparent delayed contraction observed in the primary CD8 T cell response in vaccinated newborns, relative to adult CD8 contraction (Fig. 2B), that there would also be delayed memory development (which has previously been observed in an adult infection model [62]). In consideration of the 'decreasing potential model of differentiation' [56,60], we also predicted that neonates would exhibit a more Tem biased memory development over the course of memory development. We were thus surprised to find that this was not the case. Both neonates and adults developed Tem at the same pace and ratio (relative to Tcm) post-priming (Fig. 2C). This result appeared discordant with our observation of significantly more abundant vaccine-specific CD8 T cells responses at 4-months in mice immunized as newborns (Fig. 3A), and suggests either a significantly higher level of Tcm in neonatally primed cells outside the spleen (i.e., differential memory cell trafficking) or a greater proliferative capacity of neonatally primed memory T cells. Proliferative capacities of memory CD8 T cells are programmed early in the primary immune response [49], and are dependant upon the support of APCs and CD4 T cells [63,64]. We are currently investigating the unique characteristics of the neonatally primed immune system and their effects on immune memory development.

At 2 years post-vaccination both adult and neonatally immunized mice exhibited significant expansion of vaccine-specific IFN-γ^+ T cells following in vivo challenge (Fig. 3). However, mice immunized as adults lacked CD4 T cell responses at 2 years post-immunization, while neonatally immunized mice maintained these. This may be due to different immunological environments at the time of priming. The finding that CD8 proliferation showed no reduction even up to 2 years of age suggests a fundamental difference between the longevity of CD8 and CD4 T memory T cells after priming with Lm for both age groups.

Presence of vaccine-peptide-specific IFN-γ^+ T cells is a commonly considered mechanism of protection from Lm in adult mice [35]. This relationship has not been investigated in neonatally immunized mice. We therefore attempted to examine the relevance of IFN-γ^+ T cell proliferation as a correlate of protection (CoP). We found a significant correlation between IFN-γ^+ CD8 T cell expansion and protection in both adult and neonatally immunized mice. Interestingly, the CoP—abundance of IFN-γ^+ CD8 T cells responsive to Ova—was much weaker in mice immunized as neonates than in those immunized as adults at any time point of challenge (Fig. 4). This result suggests that additional parameters contribute to the strong protection we observed in neonatally immunized mice. We previously reported that neonatal vaccination in mice elicits broader responses (co-immunodominance of several epitopes) than seen after vaccination of adults [9]; investigation of responses from a wider array of vaccine-specific CD8 T cell specificities may explain the relatively strong protective immunity in neonatally immunized mice, but relatively low CoP that we observed with one of our chosen surrogate immune markers (Ova-specific CD8 T cells). Multivalent and cross-protective T cell immunity are proposed vaccine-based solutions to pathogen heterogeneity [65] and our data suggest that vaccination near birth may improve the efficacy of these immunization strategies. We are currently in the process of delineating the contribution of breadth of epitope recognition in neonatally vs. adult immunized mice to protection.

In summary, our study is the first to identify an equivalent immune memory development in mice immunized as adults or as newborns, a near equivalent lifelong immune memory and a correlation between those memory responses and protection from infectious challenge. This strongly argues for further exploration of neonatal vaccination, given the many public health benefits that a newborn immunization strategy would bring [8,48,66]. The possibility of neonatal vaccination was demonstrated over a decade ago already, when it was shown that neonates given the 'right' stimuli would develop adult-like immune responses [67–69]. Based on our findings, *Listeria* appears to deliver the 'right' stimulation. In fact, *Listeria* is currently under investigation as a vaccine vehicle in human adults, largely due to its ability to activate a strong antigen-presenting innate immune response, leading to robust cellular adaptive immune responses [44,46]. We are cognizant of the inherent challenges of proposing a live *Listeria*-based vaccine for use with human newborns. Yet we are also hopeful that recent work performed to delineate the neonatal immune responses to experimental adjuvants [41,70] will support future research aimed at identifying the precise molecular mechanisms that endow Lm with the ability to elicit both immediate, and lifelong protective immune responses, with only one vaccine dose delivered near birth.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2010.10.013.

References

[1] Siegrist CA. Neonatal and early life vaccinology. Vaccine 2001;19:3331–46.
[2] Aspinall R, Giudice GD, Effros RB, Grubeck-Loebenstein B, Sambhara S. Challenges for vaccination in the elderly. Immun Ageing 2007;4(December (9)):11.
[3] Demirjian A, Levy O. Safety and efficacy of neonatal vaccination. Eur J Immunol 2009;39:36–46.
[4] Adkins B, Leclerc C, Marshall-Clarke S. Neonatal adaptive immunity comes of age. Nat Rev Immunol 2004;4:553–64.
[5] Siegrist CA. The challenges of vaccine responses in early life: selected examples. J Comp Pathol 2007;137(July):S4–9.
[6] WHO. The World Health Report 2005: Make Every Mother and Child Count. Geneva: WHO Press, World Health Organization; 2005.
[7] Ehreth J. The value of vaccination: a global perspective. Vaccine 2003;21:4105–17.
[8] Rodríguez-Cobo I, Chen Y-F, Olowokure B, Litchfield I. Clinical and economic assessment of different general population strategies of pertussis vaccine booster regarding number of doses and age of application for reducing whooping cough disease burden: a systematic review. Vaccine 2008;26:6768–76.
[9] Kollmann TR, Reikie B, Blimkie D, Way SS, Hajjar AM, Arispe K, et al. Induction of protective immunity to Listeria monocytogenes in neonates. J Immunol 2007;178(March (6)):3695–701.
[10] Siegrist CA. Vaccination in the neonatal period and early infancy. Int Rev Immunol 2000;19(2–3):195–219.
[11] Smith S, Jacobs RF, Wilson CB. Immunobiology of childhood tuberculosis: a window on the ontogeny of cellular immunity. J Pediatr 1997;131(July (1 Pt 1)):16–26.
[12] Dong DX, Hu XM, Liu WJ, Li JS, Jin YC, Tan SG, et al. Immunization of neonates with trivalent oral poliomyelitis vaccine (Sabin). Bull World Health Organ 1986;64(6):853–60.
[13] Wilson CB, Kollmann TR. Induction of antigen-specific immunity in human neonates and infants. Nestle Nutr Workshop Ser Pediatr Program 2008:183–95.
[14] Lewis DB, Wilson CB. Developmental immunology and role of host defenses in fetal and neonatal susceptibility to infection. In: Klein JO, Remington JS, Wilson CB, Baker CJ, editors. Infectious diseases of the fetus and newborn infant. Philiadelphia: Elsevier Saunders; 2006.
[15] Adkins B, Bu Y, Guevara P. Murine neonatal CD4+ lymph node cells are highly deficient in the development of antigen-specific Th1 function in adoptive adult hosts. J Immunol 2002;169:4998–5004.
[16] Billingham RE, Brent L, Medawar PB. Actively acquired tolerance of foreign cells. Nature 1953;172:603–6.
[17] Cihak J, Lehmann-Grube F. Immunological tolerance to lymphocytic choriomeningitis virus in neonatally infected virus carrier mice: evidence supporting a clonal inactivation mechanism. Immunology 1978;34(February (2)):265–75.
[18] Heithoff DM, Enioutina EY, Bareyan D, Daynes RA, Mahan MJ. Conditions that diminish myeloid-derived suppressor cell activities stimulate cross-protective immunity. Infect Immun 2008;76:5191–9.
[19] Siegrist CA, Aspinall R. B-cell responses to vaccination at the extremes of age. Nat Rev Immunol 2009;9(March (3)):185–94.
[20] Schatz D, Ellis T, Ottendorfer E, Jodoin E, Barrett D, Atkinson M. Aging and the immune response to tetanus toxoid: diminished frequency and level of cellular immune reactivity to antigenic stimulation. Clin Diagn Lab Immunol 1998;5(November (6)):894–6.
[21] Klein Klouwenberg P, Bont L. Neonatal and infantile immune responses to encapsulated bacteria and conjugate vaccines. Clin Dev Immunol 2008;2008:628963.
[22] Carbone FR, Moore MW, Sheil JM, Bevan MJ. Induction of cytotoxic T lymphocytes by primary in vitro stimulation with peptides. J Exp Med 1988;167(June (6)):1767–79.
[23] Geginat G, Schenk S, Skoberne M, Goebel W, Hof H. A novel approach of direct ex vivo epitope mapping identifies dominant and subdominant CD4 and CD8 T cell epitopes from Listeria monocytogenes. J Immunol 2001;166(February (3)):1877–84.
[24] Yefenof E, Zehavi-Feferman R, Guy R. Control of primary and secondary antibody responses by cytotoxic T lymphocytes specific for a soluble antigen. Eur J Immunol 1990;20(August (8)):1849–53.
[25] Way SS, Kollmann TR, Hajjar AM, Wilson CB. Protective cell-mediated immunity to listeria monocytogenes in the absence of myeloid differentiation factor 88. J Immunol 2003;171:533–7.
[26] Lee J, Spidlen J, Boyce K, Cai J, Crosbie N, Dalphin M, et al. MIFlowCyt: the minimum information about a flow cytometry experiment. Cytometry A 2008;73(October (10)):926–30.
[27] Smolen KK, Loeffler DI, Reikie BA, Aplin L, Cai B, Fortuno 3rd ES, et al. Neonatal immunization with Listeria monocytogenes induces T cells with an adult-like avidity, sensitivity, and TCR-Vbeta repertoire, and does not adversely impact the response to boosting. Vaccine 2009;28(December (1)):235–42.
[28] Loeffler DI, Smolen K, Aplin L, Cai B, Kollmann TR. Fine-tuning the safety and immunogenicity of Listeria monocytogenes-based neonatal vaccine platforms. Vaccine 2009;27:919–27.
[29] Porter BB, Harty JT. The onset of CD8+-T-cell contraction is influenced by the peak of Listeria monocytogenes infection and antigen display. Infect Immun 2006;74:1528–36.
[30] Starks H, Bruhn KW, Shen H, Barry RA, Dubensky TW, Brockstedt D, et al. Listeria monocytogenes as a vaccine vector: virulence attenuation or existing antivector immunity does not diminish therapeutic efficacy. J Immunol 2004;173(July (1)):420–7.
[31] Huster KM, Koffler M, Stemberger C, Schiemann M, Wagner H, Busch DH. Unidirectional development of CD8+ central memory T cells into protective Listeria-specific effector memory T cells. Eur J Immunol 2006;36:1453–64.
[32] Onami TM, Harrington LE, Williams MA, Galvan M, Larsen CP, Pearson TC, et al. Dynamic regulation of T cell immunity by CD43. J Immunol 2002;168(June (12)):6022–31.
[33] Masopust D, Ha SJ, Vezys V, Ahmed R. Stimulation history dictates memory CD8 T cell phenotype: implications for prime-boost vaccination. J Immunol 2006;177(July (2)):831–9.
[34] Siegrist CA, Saddallah F, Tougne C, Martinez X, Kovarik J, Lambert PH. Induction of neonatal TH1 and CTL responses by live viral vaccines: a role for replication patterns within antigen presenting cells? Vaccine 1998;16(August–September (14–15)):1473–8.
[35] Hamilton SE, Porter BB, Messingham KA, Badovinac VP, Harty JT. MHC class Ia-restricted memory T cells inhibit expansion of a nonprotective MHC class Ib (H2-M3)-restricted memory response. Nat Immunol 2004;5:159–68.
[36] Plotkin SA. Vaccines: correlates of vaccine-induced immunity. Clin Infect Dis 2008;47(August (3)):401–9.
[37] McElhaney JE, Xie D, Hager DW, Barry MB, Wang Y, Kleppinger A, et al. T cell responses are better correlates of vaccine protection in the elderly. J Immunol 2006;176:6333–9.
[38] Govaert TM, Sprenger MJ, Dinant GJ, Aretz K, Masurel N, Knottnerus JA. Immune response to influenza vaccination of elderly people. A randomized double-blind placebo-controlled trial. Vaccine 1994;12(13):1185–9.
[39] Whittle HC, Aaby P, Samb B, Jensen H, Bennett J, Simondon F. Effect of subclinical infection on maintaining immunity against measles in vaccinated children in West Africa. Lancet 1999;353:98–102.
[40] Li L, Lee HH, Bell JJ, Gregg RK, Ellis JS, Gessner A, et al. IL-4 utilizes an alternative receptor to drive apoptosis of Th1 cells and skews neonatal immunity toward Th2. Immunity 2004;20(April (4)):429–40.
[41] Kollmann TR, Crabtree J, Rein-Weston A, Blimkie D, Thommai F, Wang XY, et al. Neonatal innate TLR-mediated responses are distinct from those of adults. J Immunol 2009;183:7150–60.
[42] Cohen JJ, Rodriguez GE, Kind PD, Campbell PA. Listeria cell wall fraction: a B cell mitogen. J Immunol 1975;114(March (3)):1132–4.
[43] Zenewicz LA, Shen H. Innate and adaptive immune responses to Listeria monocytogenes: a short overview. Microbes Infect 2007;9(August (10)):1208–15.
[44] Maciag PC, Radulovic S, Rothman J. The first clinical use of a live-attenuated Listeria monocytogenes vaccine: A phase I safety study of Lm-LLO-E7 in patients with advanced carcinoma of the cervix. Vaccine 2009;27(June (30)):3975–83.
[45] Wood LM, Guirnalda PD, Seavey MM, Paterson Y. Cancer immunotherapy using Listeria monocytogenes and listerial virulence factors. Immunol Res 2008;42(1–3):233–45.
[46] Wallecha A, Maciag PC, Rivera S, Paterson Y, Shahabi V. Construction and characterization of an attenuated Listeria monocytogenes strain for clinical use in cancer immunotherapy. Clin Vaccine Immunol 2009;16(January (1)):96–103.
[47] Lee SM, Suen Y, Chang L, Bruner V, Qian J, Indes J, et al. Decreased interleukin-12 (IL-12) from activated cord versus adult peripheral blood mononuclear cells and upregulation of interferon-gamma, natural killer, and lymphokine-activated killer activity by IL-12 in cord blood mononuclear cells. Blood 1996;88:945–54.
[48] Roduit C, Bozzotti P, Mielcarek N, Lambert P-H, Giudice Gd, Locht C, et al. Immunogenicity and protective efficacy of neonatal vaccination against Bordetella pertussis in a murine model: evidence for early control of pertussis. Infect Immun 2002;70:3521–8.
[49] Badovinac VP, Porter BB, Harty JT. Programmed contraction of CD8(+) T cells after infection. Nat Immunol 2002;3(July (7)):619–26.
[50] Regner M, Culley F, Fontannaz P, Hu K, Morein B, Lambert PH, et al. Safety and efficacy of immune-stimulating complex-based antigen delivery systems for neonatal immunisation against respiratory syncytial virus infection. Microbes Infect 2004;6(June (7)):666–75.
[51] Jakobsen H, Bjarnarson S, Giudice GD, Moreau M, Siegrist CA, Jonsdottir I. Intranasal immunization with pneumococcal conjugate vaccines with LT-K63, a nontoxic mutant of heat-Labile enterotoxin, as adjuvant rapidly induces protective immunity against lethal pneumococcal infections in neonatal mice. Infect Immun 2002;70(March (3)):1443–52.
[52] VanCott JL, Prada AE, McNeal MM, Stone SC, Basu M, Huffer BJ, et al. Mice develop effective but delayed protective immune responses when immunized as neonates either intranasally with nonliving VP6/LT(R192G) or orally with live rhesus rotavirus vaccine candidates. J Virol 2006;80(May (10)):4949–61.
[53] Weng N-P. Aging of the immune system: how much can the adaptive immune system adapt? Immunity 2006;24(May):495–9.

[54] Kang I, Hong MS, Nolasco H, Park SH, Dan JM, Choi Y, et al. Age-associated change in the frequency of memory CD4+ T cells impairs long term CD4+ T cell responses to influenza vaccine. J Immunol 2004;173(July (1)):673–81.
[55] Vekemans J, Ota MO, Wang EC, Kidd M, Borysiewicz LK, Whittle H, et al. T cell responses to vaccines in infants: defective IFNgamma production after oral polio vaccination. Clin Exp Immunol 2002;127(March (3)):495–8.
[56] Kalia V, Sarkar S, Gourley TS, Rouse BT, Ahmed R. Differentiation of memory B and T cells. Curr Opin Immunol 2006;18(June (3)):255–64.
[57] Roberts AD, Ely KH, Woodland DL. Differential contributions of central and effector memory T cells to recall responses. J Exp Med 2005;202(July (1)):123–33.
[58] Hogan RJ, Cauley LS, Ely KH, Cookenham T, Roberts AD, Brennan JW, et al. Long-term maintenance of virus-specific effector memory CD8+ T cells in the lung airways depends on proliferation. J Immunol 2002;169(November (9)):4976–81.
[59] Hammarlund E, Lewis MW, Hansen SG, Strelow LI, Nelson JA, Sexton GJ, et al. Duration of antiviral immunity after smallpox vaccination. Nat Med 2003;9(September (9)):1131–7.
[60] Joshi NS, Kaech SM. Effector CD8 T cell development: a balancing act between memory cell potential and terminal differentiation. J Immunol 2008;180(February (3)):1309–15.
[61] Hikono H, Kohlmeier JE, Takamura S, Wittmer ST, Roberts AD, Woodland DL. Activation phenotype, rather than central- or effector-memory phenotype, predicts the recall efficacy of memory CD8+ T cells. J Exp Med 2007;204(July (7)):1625–36.
[62] Badovinac VP, Harty JT. Manipulating the rate of memory CD8+ T cell generation after acute infection. J Immunol 2007;179(July (1)):53–63.
[63] Obar JJ, Molloy MJ, Jellison ER, Stoklasek TA, Zhang W, Usherwood EJ, et al. CD4+ T cell regulation of CD25 expression controls development of short-lived effector CD8+ T cells in primary and secondary responses. Proc Natl Acad Sci U S A 2010;107(January (1)):193–8.
[64] Livingstone AM, Wilson EB, Ontiveros F, Wang J-CE. Unravelling the mechanisms of help for CD8+ T cell responses. Immunol Res 2009;45:209–17.
[65] Botten J, Sidney J, Mothe BR, Peters B, Sette A, Kotturi MF. Coverage of related pathogenic species by multivalent and cross-protective vaccine design: arenaviruses as a model system. Microbiol Mol Biol Rev 2010;74(June (2)):157–70.
[66] Kovarik J, Siegrist CA. Optimization of vaccine responses in early life: the role of delivery systems and immunomodulators. Immunol Cell Biol 1998;76:222–36.
[67] Forsthuber T, Yip HC, Lehmann PV. Induction of TH1 and TH2 immunity in neonatal mice. Science 1996;271(March (5256)):1728–30.
[68] Ridge JP, Fuchs EJ, Matzinger P. Neonatal tolerance revisited: turning on newborn T cells with dendritic cells. Science 1996;5256(March (271)):1723–6.
[69] Sarzotti M, Robbins DS, Hoffman PM. Induction of protective CTL responses in newborn mice by a murine retrovirus. Science 1996;271(March (5256)):1726–8.
[70] Nguyen M, Leuridan E, Zhang T, De Wit D, Willems F, Van Damme P, et al. Acquisition of adult-like TLR4 and TLR9 responses during the first year of life. PLoS ONE 2010;5(4):e10407.