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# Induction of Protective Immunity to *Listeria monocytogenes* in Neonates<sup>1</sup>

Tobias R. Kollmann,<sup>2\*†‡</sup> Brian Reikie,<sup>‡</sup> Darren Blimkie,<sup>‡</sup> Sing Sing Way,<sup>\*†</sup> Adeline M. Hajjar,<sup>\*</sup> Kiea Arispe,<sup>\*</sup> Angela Shaulov,<sup>\*</sup> and Christopher B. Wilson<sup>\*†</sup>

Neonates suffer unduly from infections and also respond suboptimally to most commonly used vaccines. However, a CD8 T cell response can be elicited in neonates if the Ag is introduced into the cytoplasm of APCs. *Listeria monocytogenes* (Lm) targets the cytoplasm of APC and is a strong CD8 and CD4 Th1-promoting vaccine vehicle in adult mice. We hypothesized that an attenuated strain of Lm would be safe and induce long-lasting protective immunity, even in neonates. We found that neonatal mice immunized only once with the attenuated strain  $\Delta actA$ -Lm developed robust primary and secondary CD8 and CD4 Th1 responses and were fully protected from lethal challenge with virulent wild-type Lm without the need for a booster immunization. Furthermore,  $\Delta actA$ -Lm expressing a heterologous recombinant Ag induced a strong CD8 and Th1 memory response to that Ag. Based on these data, we propose that  $\Delta actA$ -Lm or derivatives thereof might serve as a vaccine vehicle for neonatal immunization. *The Journal of Immunology*, 2007, 178: 3695–3701.

Approximately 2.5 million neonates and infants die annually from infection, marking this as the time of life most burdened by infectious diseases (1, 2). Newborns are especially prone to infections for which cell-mediated immunity is protective (1, 3). Neonates and infants also have a reduced or suboptimal capacity to mount an effective cell-mediated immunity in response to most vaccines (1, 3–5). However, introducing a vaccine Ag into the cytoplasm of APCs induces a significant CD8 CTL response even in neonates (3, 6, 7). Contrary to the induction of CTLs, Th1 (IFN- $\gamma$  producing) CD4 T cell responses have not only been difficult to induce in the neonate, but appear even more difficult to sustain (4, 5, 8, 9).

*Listeria monocytogenes* (Lm)<sup>3</sup> is a Gram-positive microbe that resides primarily in the intracytoplasmic compartment of host cells, including the primary APCs, such as macrophages and dendritic cells. Furthermore, Lm possesses biologically strong Th1 adjuvant characteristics, making it an attractive vehicle for vaccinations (10, 11). Recombinant attenuated strains of Lm induce specific immunity, even in the presence of preexisting immunity to the Lm vehicle (10), making Lm an ideal candidate vector for neonatal vaccination in the face of preexisting maternal immunity.

But Lm is also a serious pathogen in neonatal life, precluding its use for vaccination without further study. We hypothesized that an attenuated strain of Lm could trigger an Ag-specific and protective memory immune response in the neonate. We found that Lm mutants with the targeted deletion in the virulence determinant ActA ( $\Delta actA$ -Lm) induce a strong primary and secondary Th1 CD4 and CD8 T cell response in neonatally immunized mice, and provide sterilizing protection from lethal challenge after only a single immunization. Furthermore,  $\Delta actA$ -Lm expressing a heterologous Ag allowed induction of an Ag-specific memory T cell response to the heterologous Ag, suggesting attenuated strains of Lm might serve as a vehicle for neonatal vaccination.

## Materials and Methods

### Mice

Because Lm class I immunodominant peptides have been described only in the murine H-2<sup>d</sup> haplotype and class II immunodominant peptides only in the H-2<sup>b</sup> haplotype, we used neonatal and 6-wk-old adult F<sub>1</sub> mice (H-2<sup>b</sup> × H-2<sup>d</sup>) derived from matings between C57BL/6 (H-2<sup>b</sup>) and C57B10.D2 (H-2<sup>d</sup>) mice (12). In preliminary experiments, there was no difference in the Lm-specific T cell responses in C57BL/6 (H-2<sup>b</sup>) or C57B10.D2 (H-2<sup>d</sup>) compared with F<sub>1</sub> (H-2<sup>b</sup> × H-2<sup>d</sup>) mice for the respective immunodominant epitopes (data not shown). Because our experiments were meant to provide insight into mechanisms possibly underlying the human neonate's reduced response to vaccines, we chose the 5- to 7-day-old murine pup, since they are the closest to human neonates with respect to the maturational status of their immune system (1).

### Bacterial strains and infection

Wild-type (WT) Lm strain 10403s,  $\Delta actA$  strain DPL1942, and Lm-OVA were provided by Dr. D. Portnoy (University of California, Berkeley, CA), Dr. N. Freitag (Seattle Biomedical Research Institute, Seattle, WA), and Dr. H. Shen (University of Pennsylvania, Philadelphia, PA), respectively.  $\Delta actA$ -Lm-OVA was constructed from Lm-OVA by cloning ~500 bp fragments of the *actA* gene into the *HindIII/KpnI* sites of the temperature-sensitive plasmid pKSV7 using the following primers: upstream flanking region, forward primer 5'-aagcttgacgacgacgatagcgaag-3', reverse primer 5'-gaattccgctgcgctatccgatgg-3', downstream flanking region, forward primer 5'-gaattcgttaagtccaaggtatcg-3', reverse primer 5'-ggtaccctaaagagaa cagccaatag-3' (underlined sequences indicate introduced restriction sites). All strains were grown as described previously (13). In brief, Lm were grown to mid-log phase (OD<sub>600</sub>, 0.1) at 37°C, diluted in 100  $\mu$ l of saline, and injected i.p. into mice for immunization or i.v. in 200  $\mu$ l of saline for challenge. The number of Lm in lysates of infected spleens and livers was

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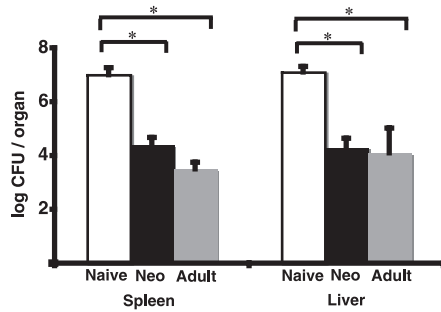
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<sup>3</sup> Abbreviations used in this paper: Lm, *Listeria monocytogenes*; WT, wild type.

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**FIGURE 1.** Neonatally immunized mice were protected from WT Lm challenge to the same degree as mice immunized as adults. Mice immunized with  $1 \times 10^4$  CFU i.p. of  $\Delta actA$ -Lm on day 5 of life (Neo, ■) or at 6 wk of age (Adult, ▨), or age-matched nonimmune (Naive, □) mice were infected i.v. with  $1 \times 10^5$  CFU of WT Lm 3 mo after immunization. Lm CFUs in the spleens or livers of mice 3 days after infection were determined and shown is the mean from a total of 12 mice/group combined from three separate experiments. Bar, SE; \*, Statistically significant,  $p < 0.05$ .

determined as described previously (13), with a lower limit of detection of 20 CFU and 30 CFU/organ for spleen and liver, respectively. All experiments were performed under Institutional Animal Care and Use Committee-approved protocols.

#### Enumeration of Ag-specific T cells

Intracellular cytokine staining was performed as described (13) using reagents from BD Pharmingen. In brief, splenocyte suspensions were prepared by homogenizing spleens between two sterile glass slides, subjected to RBC lysis, and filtered through a 70- $\mu$ m mesh. A total of  $2.0 \times 10^6$  splenocytes was incubated for 5 h in 200  $\mu$ l of RPMI 1640 medium supplemented with 10% FCS (HyClone), L-glutamine, penicillin, and streptomycin in the presence of the indicated Lm- or OVA-specific peptide ( $10^{-6}$  M) and GolgiStop. Cells were stained for 30 min on ice with FITC-labeled CD3 and either PerCP-labeled anti-CD8 or anti-CD4. For surface tetramer staining, tetramers (National Institute of Allergy and Infectious Diseases Tetramer Facility) specific for the LLO<sub>91-99</sub> or P60<sub>217-225</sub> epitope were used. To detect intracellular cytokine production, cells were permeabilized with Cytoperm solution and stained with allophycocyanin-labeled anti-IFN- $\gamma$  and PE-labeled anti-IL-4 for 30 min at room temperature after surface staining as described above. Stained cells were acquired on a FACSCanto flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

#### Cytokine production

The concentration of IL-4 and IL-13 in the supernatants of splenocyte cultures was determined 72 h after peptide stimulation by ELISA using reagents from R&D Systems as previously described (13).

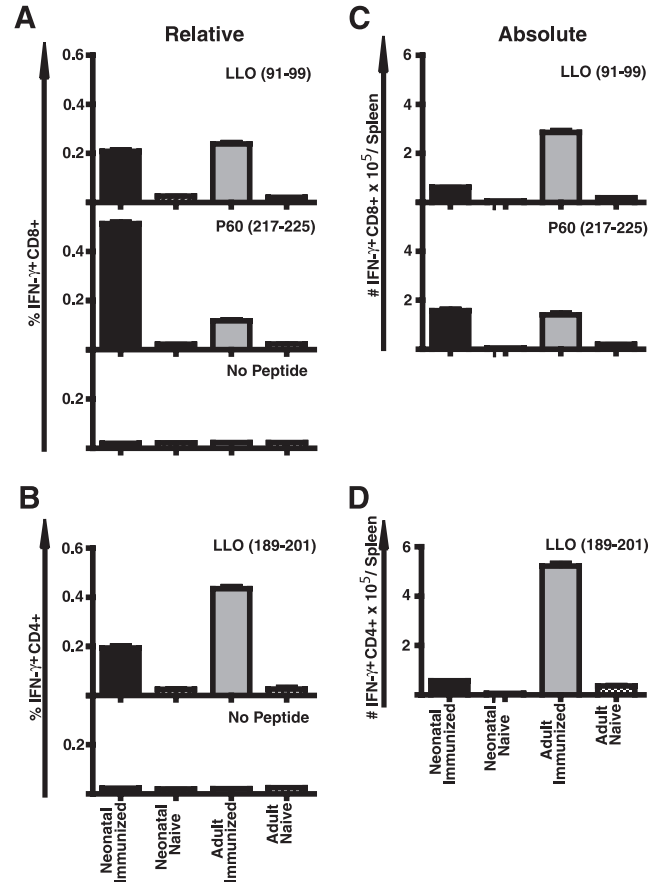
#### Statistics

The differences in geometric mean CFUs, the percentages and numbers of activated splenocytes, and the cytokine concentrations were evaluated using the Student's *t* test, with  $p < 0.05$  taken as statistically significant.

## Results

### Attenuated strains of Lm are safe in neonates

The listerial *actA* gene is required for polymerization of host actin into filaments, which allows cell-cell spread using components of the host cell's actin cytoskeleton. The  $\Delta actA$  strain of Lm carries an in-frame deletion in the *actA* gene ( $\Delta actA$ -Lm), is unable to spread from cell-cell, and displays a three order of magnitude decrease in virulence without interfering with immunogenicity (10, 14). We and others have shown that  $\Delta actA$ -Lm was safe in MyD88-deficient or IFN- $\gamma$ -deficient adult mice, which, similar to neonates, are highly susceptible to Lm infection (13, 15–17). We found that neonatal mice survived high-dose infection with  $\Delta actA$ -Lm without any sign of disease. Although the LD<sub>50</sub> of neonates for WT Lm is  $\sim 10^1$  CFU, we found that the LD<sub>50</sub> for

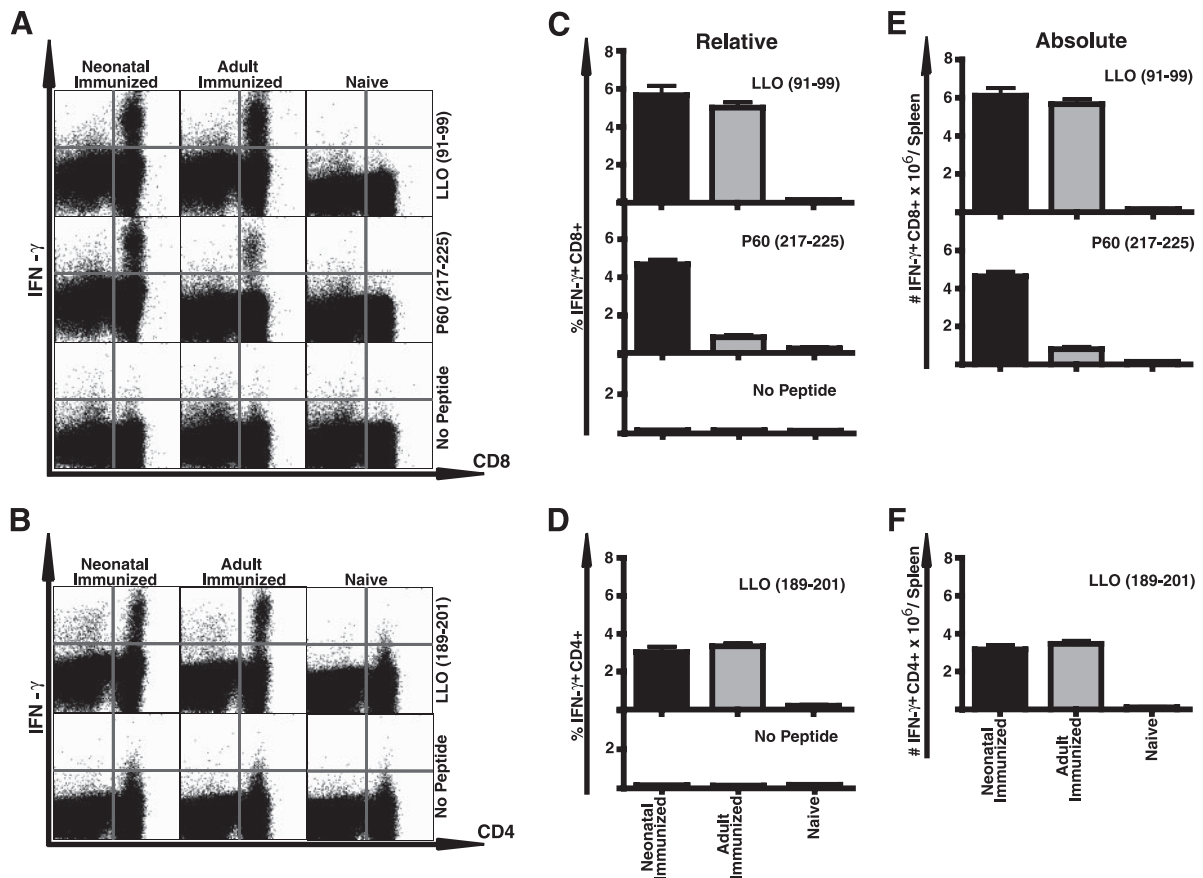


**FIGURE 2.** Neonatally immunized mice develop an Lm-specific primary CD8 and CD4 T cell response. Spleen cells were obtained from the indicated mice 7 days after infection with  $\Delta actA$ -Lm and stimulated with the indicated MHC class I- or class II-restricted Lm-specific peptides before analysis of intracellular cytokine expression by flow cytometry. Shown is the mean percentage (A and B) or total number per spleen (C and D) of CD8 (A and C) or CD4 (B and D) IFN- $\gamma$ -producing splenocytes. For each group, the percentage of unstimulated IFN- $\gamma$ -producing control cells is shown for CD4 or CD8, and the numbers of these cells were subtracted from the absolute numbers of Ag-specific cells shown (C and D). These data represent five mice per group from two combined experiments. Bar, SE.

$\Delta actA$ -Lm is between  $10^6$  and  $10^7$  CFU. Examination of spleen and liver homogenates of neonatal or adult mice 7 days after vaccination with up to  $10^6$  CFU  $\Delta actA$ -Lm revealed no recoverable bacteria. Thus,  $\Delta actA$ -Lm was safe and well tolerated in neonates.

### Neonates mount a protective immune response similar to adults

Immunization of adult mice with  $\Delta actA$ -Lm confers protection against secondary Lm infection (18). To address whether  $\Delta actA$ -Lm does so in neonates as well, mice were immunized with  $10^4$  CFU  $\Delta actA$ -Lm at 5 days of age (neonatal immunization) or at 6 wk of age (adult immunization). Both groups of mice were challenged 3 mo later with an inoculum of WT Lm that would be lethal for naive adult mice ( $10^5$  CFU). Three days after challenge, the numbers of Lm in the spleens and livers of mice immunized as neonates were dramatically reduced compared with naive age-matched controls (Fig. 1). Similar to mice immunized as adults, all neonatally immunized mice remained healthy, survived, and eventually cleared the infection completely by 10 days postchallenge. In contrast, bacterial replication continued in naive control mice, which became moribund, and had to be sacrificed by day 4. Thus,



**FIGURE 3.** Neonatally immunized mice develop an Lm-specific secondary CD8 and CD4 T cell response. Mice immunized with  $1 \times 10^4$  CFU i.p. of  $\Delta actA$ -Lm on day 5 of life (Neonate) or at 6 wk of age (Adult) were infected i.v. with  $1 \times 10^5$  CFU of WT Lm 3 mo after immunization. Spleen cells were obtained from indicated mice 5 days after infection along with age-matched nonimmune (Naive) splenocytes and stimulated with the indicated MHC class I- or class II-restricted Lm-specific peptides before analysis of intracellular cytokine expression by flow cytometry. Shown in *A* for CD8 and in *B* for CD4 are examples of the flow cytometric analysis, and in the other panels the mean percentage (*C* and *D*) and total number per spleen (*E* and *F*) of CD8 (*C* and *E*) or CD4 (*D* and *F*) IFN- $\gamma$ -producing splenocytes are shown. For each group, unstimulated IFN- $\gamma$ -producing controls are shown in the examples, as well as in the summary graphs for CD4 or CD8, and the numbers of these cells were subtracted from the absolute numbers of Ag-specific cells shown (*C*–*F*). The data in *C*–*F* represent five mice per group from two combined experiments. Bar, SE.

neonatal immunization, like adult immunization, resulted in protective sterilizing immunity to subsequent challenge with WT Lm.

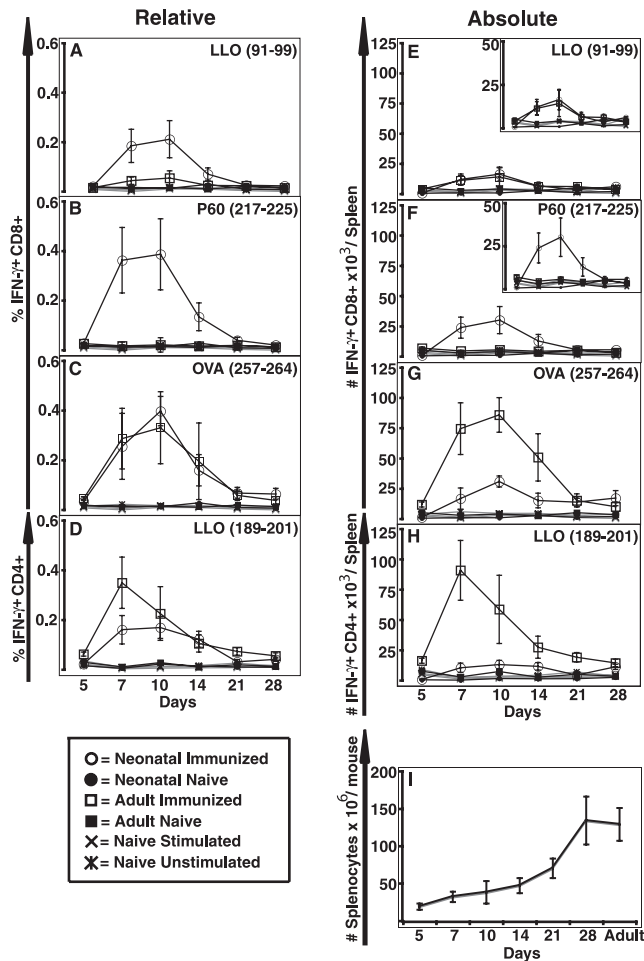
#### Neonates mount a primary T cell response similar to adults

To our knowledge, there are no published reports on the successful induction of Ag-specific CD8 or CD4 primary T cells in response to Lm infection in the neonate. Seven days after immunization with  $\Delta actA$ -Lm, we enumerated Lm-specific CD8 and CD4 T cells in neonatal and adult mice by intracellular IFN- $\gamma$  (a marker for a Th1-type response) and IL-4 (a marker for a Th2-type response) staining of splenocytes after restimulation *in vitro* with Lm-specific MHC class I listeriolysin O (LLO)<sub>91–99</sub>, P60<sub>217–225</sub>, P60<sub>449–457</sub> and MP<sub>184–192</sub>, or MHC class II LLO<sub>189–201</sub>-restricted peptides (Fig. 2). The neonate generated similar percentages of LLO<sub>91–99</sub>-specific CD8 T cells compared with adult mice and 3-fold higher percentages of P60<sub>217–225</sub>-specific CD8 T cells than adult mice. This response could be detected by intracellular IFN- $\gamma$  staining (Fig. 2) or by surface staining with tetramers specific for LLO or P60 (data not shown). Detection of the subdominant epitopes P60<sub>449–457</sub> and MP<sub>184–92</sub> was similarly low in both adult and neonate (data not shown). A substantial percentage of neonatal CD4 T cells produced IFN- $\gamma$  in response to LLO<sub>189–201</sub> peptide stimulation, although 2-fold less than adult cells. With only  $\sim 30 \times 10^6$  cells/spleen in the 12-day-old, but  $\sim 120 \times 10^6$  cells/spleen in the

adult mice, the absolute numbers of LLO-specific CD8 (Fig. 2C) and CD4 (Fig. 2D) T cells per spleen were lower in mice immunized as neonate vs adult, but the absolute numbers of P60-specific CD8 T cells were equivalent (Fig. 2C). Neither neonatally nor adult-immunized mice displayed a CD4 Th2-type response as measured by IL-4 production in a flow cytometric assay or IL-4 or IL-13 production by ELISA (data not shown). Thus, neonates similar to adults were able to mount a CD8 and CD4 Th1 primary response.

#### Neonates mount a secondary T cell response similar to adults

Sterilizing immunity to Lm in adults is mediated by Ag-specific T cells. Our data suggested that neonatal immunization with  $\Delta actA$ -Lm induced T cell responses similar to adults. To test this directly, we immunized neonates and adults and, 3 mo later, enumerated Lm-specific CD8 and CD4 T cells by intracellular IFN- $\gamma$  staining 5 days after secondary infection (Fig. 3). Neonatally and adult-immunized mice generated equivalent relative and absolute numbers of LLO<sub>91–99</sub>-specific CD8 T cells. Neonatally immunized mice mounted an  $\sim 3$ -fold higher P60<sub>217–225</sub>-specific CD8 T cell response than mice immunized as adults. This finding was confirmed using LLO- and P60-specific tetramers (data not shown). Detection of the subdominant epitopes P60<sub>449–457</sub> and MP<sub>184–92</sub> again was similarly low in both adult and neonatally immunized



**FIGURE 4.** Neonatally immunized mice develop an Lm- and OVA-specific primary CD8 T cell response. Spleen cells were obtained from the indicated mice 5, 7, 10, 14, 21, or 28 days after infection with  $\Delta actA$ -Lm-OVA and stimulated with the indicated MHC class I-restricted Lm- or OVA-specific peptides before analysis of intracellular cytokine expression by flow cytometry. Shown are the mean percentage (A–D) and total number per spleen (E–H) of CD8 (A–C and E–G) or CD4 (D and H) IFN- $\gamma$ -producing splenocytes. Naive age-matched mice and unstimulated controls are included in each graph. The inset in E and F show the same data, but on a smaller scale than the main figure for better visualization. I, Total number of splenocytes for each age group. These data represent 5–10 mice per age group from two combined experiments. Bar, SE.

mice (data not shown). Contrary to what was seen for the primary CD4 T cell response, neonatally immunized mice mounted as effective a CD4 memory response as mice immunized as adults. Importantly, there was no reversion to a Th2 phenotype, because in response to Lm peptide stimulation neither in mice immunized as neonates nor as adults were IL-4-producing CD4 T cells observed by flow cytometry, and both neonatal or adult CD4 T cells made similar low levels of IL-4 or IL-13 as measured by ELISA (data not shown). Neonates thus mount a sustained CD4 Th1 response along with a strong CD8 T cell memory response.

#### *The neonate and adult display similar primary immune response kinetics to $\Delta actA$ -Lm expressing heterologous Ag*

Given its ability to induce a strong, sustained protective Th1 memory response, we asked whether  $\Delta actA$ -Lm could function as a vaccine vehicle in the neonate. To test this, we generated  $\Delta actA$ -Lm-OVA, which expresses the known dominant MHC I-restricted CD8 T cell epitope of OVA, and immunized neonates and

adults with this recombinant strain. As shown in Fig. 4, the adult CD8 T cell primary response was skewed to the now dominant OVA<sub>257–264</sub> epitope of OVA, with the endogenous Lm LLO<sub>91–99</sub> and P60<sub>217–225</sub> CD8 T cell response detected only at background levels. Contrary to the adult, the  $\Delta actA$ -Lm-OVA-immunized neonate developed not only a substantial OVA<sub>257–264</sub> CD8 T cell response, but maintained a substantial fraction of LLO<sub>91–99</sub> and P60<sub>217–225</sub>-reactive CD8 T cells. Expansion, peak, and contraction of Ag-specific T cells followed similar kinetics in mice immunized as adults or as neonates (Fig. 4). The peak absolute number of OVA<sub>257–264</sub>-specific CD8 T cells was lower in mice immunized as neonate vs adult (Fig. 4G). But the peak absolute numbers of LLO<sub>91–99</sub> were equal (Fig. 4E), and the peak absolute numbers of P60<sub>217–225</sub>-specific (Fig. 4F) CD8 T cells were even higher in the neonatally immunized mice at all time points examined. Although the total number of cells per spleen not unexpectedly differs between 12-day-old and ~8-wk-old mice (see Fig. 4I), there was no significant difference in total cell number per spleen between immunized or naive mice within each age group ( $n = 12$ /group; data not shown). At 28 days after immunization, when the contraction phase was complete and neonatally or adult-immunized mice had equivalent total spleen cell numbers, neonatally immunized mice contained low but equivalent, if not greater, absolute numbers of Ag-specific CD8 and CD4 memory T cells as compared with adult immune mice (Fig. 5).

#### *Neonates mount a memory T cell response to $\Delta actA$ -Lm expressing a heterologous Ag*

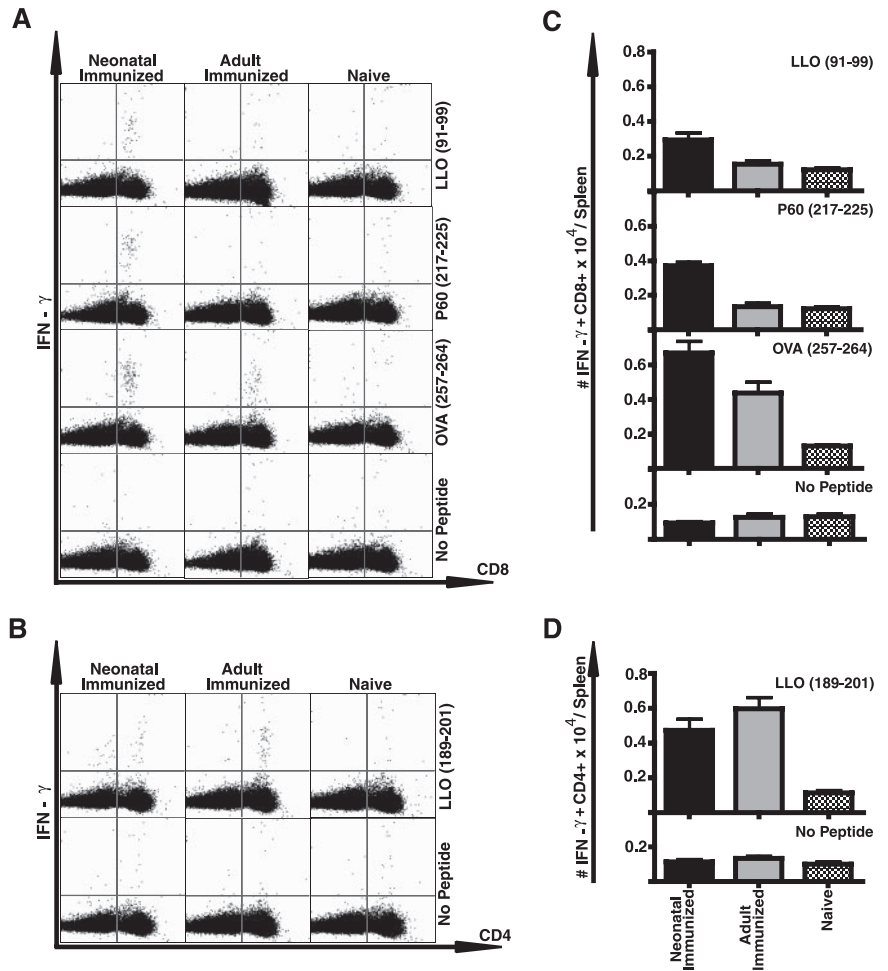
Mice immunized with  $\Delta actA$ -Lm-OVA as neonates or adults were challenged 3 mo later with WT Lm-expressing OVA. Five days after secondary infection, we enumerated Ag-specific T cells by intracellular IFN- $\gamma$  staining in response to peptide restimulation. As shown in Fig. 6, the same relative CD8 T cell epitope hierarchy as detected in the primary response was maintained in both adult and neonatally immunized mice, with the adult CD8 T cell memory response completely dominated by the OVA<sub>257–264</sub> response, but the neonatally immunized mice displaying not only significant OVA<sub>257–264</sub> responses, but also LLO<sub>91–99</sub> and P60<sub>217–225</sub>-reactive CD8 T cells responses. In summary, neonates mounted a strong primary and secondary memory CD8 T cell response to the heterologous OVA Ag.

## Discussion

We set out to test whether the attenuated strain  $\Delta actA$ -Lm could induce a protective immune response in mice immunized as neonates. In summary, we found the following: immunization with  $\Delta actA$ -Lm provided long-lasting protection after only one dose given around birth; immunization with  $\Delta actA$ -Lm induced a primary T cell response with similar kinetics in neonatal and adult mice; neonatally immunized mice developed robust and sustained Th1 CD4 and CD8 Lm-specific T cell memory responses after only a single round of immunization; and  $\Delta actA$ -Lm expressing a heterologous Ag (OVA) induced a strong Ag-specific primary and memory CD8 T cell response. Based on these findings, we propose that  $\Delta actA$ -Lm (or a derivative thereof) might provide a suitable platform for neonatal vaccination.

Protection against intracellular pathogens appears significantly limited in the newborn. Recent evidence indicates that, under appropriate conditions, human and murine neonates can mount a detectable CD8 T cell immune response (1–3). The key requirement for a successful induction of neonatal CD8 T cell response in the mouse appears to be the entrance of the Ag into the cytoplasm of APCs (3, 6, 7, 19). Lm fulfills this requirement (10, 11). We thus hypothesized that Lm might serve as a neonatal vaccine vehicle.

**FIGURE 5.** Neonatally immunized mice develop an Lm- and OVA-specific memory T cell response. Spleen cells were obtained from the indicated mice after completion of the contraction phase at 28 days after infection with  $\Delta actA$ -Lm-OVA and stimulated with the indicated MHC class I-restricted Lm- or OVA-specific peptides before analysis of intracellular cytokine expression by flow cytometry. Shown in *A* for CD8 and in *B* for CD4 are examples of the flow cytometric analysis; the other panels show the mean total number per spleen of CD8 (*C*) or CD4 (*D*) IFN- $\gamma$ -producing splenocytes. For each group, unstimulated IFN- $\gamma$ -producing controls are shown in the examples, as well as in the summary graphs for CD4 or CD8. The data represent five mice per group. Bar, SE.

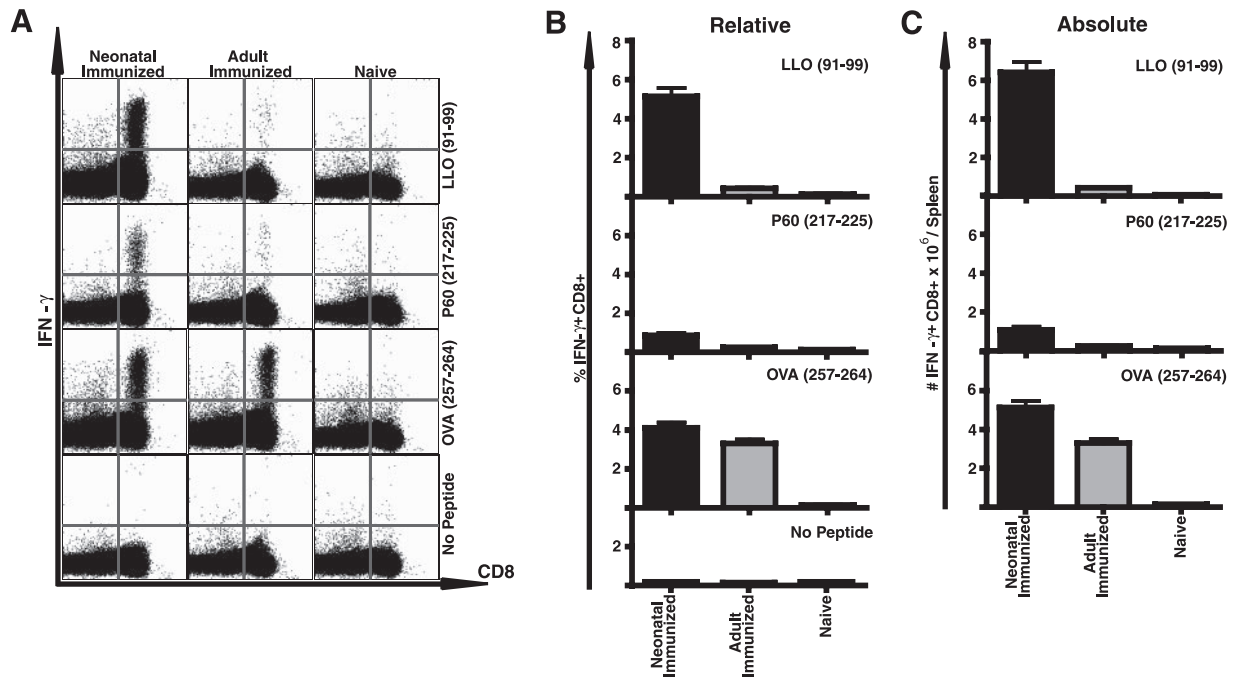


Unfortunately, very little is known about the immune response to *Listeria* in either the murine or the human neonate. Although survival of neonatal mice infected with Lm can be increased through pretreatment with Flt-3 ligand, anti-IL-10 Ab, or CpG adjuvant (20–22), the natural resistance to Lm infection only slowly increases in murine pups to reach adult levels around 4 wk of age (23). To address this increased susceptibility of neonates to Lm infection, a previous study examined a hyperattenuated auxotrophic strain of Lm, but this strain does not induce a detectable memory CD8 T cell response unless mice are boosted as adults (24). We have shown that the  $\Delta actA$  strain of Lm is safe and induces primary as well as secondary CD4 Th1 and CD8 T cell responses in adult MyD88-deficient mice, which are highly susceptible to infection with WT Lm (15). We now show that neonatal mice survived a high dose of  $\Delta actA$ -Lm infection without any sign of disease, and cleared the inoculum similar to adult mice. Although  $\Delta actA$ -Lm derivatives have been shown to be safe in adult mice (10, 11), ours is the first demonstration of their safety in neonatal mice.

It is known that neonatal mice and humans develop functional CD8 T cells after viral infection (1, 3), but nothing at all is known about the neonatal primary response to Lm. We show here that neonatal mice, after only a single round of immunization with  $\Delta actA$ -Lm, developed not only a strong CD8, but also a substantial CD4 Th1 primary T cell response. This, to our knowledge, is the first time a single immunization given to a neonatal mouse was able to induce strong CD8 and CD4 Th1 primary T cell response. More importantly, we found that a single immunization with  $\Delta actA$ -Lm was sufficient to induce long-lasting protection from

challenge with an otherwise lethal inoculum of WT Lm. Only the survivors of neonatal mice pretreated with CpG before Lm challenge had previously been shown to develop a protective memory response (20). Our data indicate that  $\Delta actA$ -Lm activates mechanisms leading to sustained CD4 and CD8 T cell memory responses that are fully functional in the neonatal mouse.

Recombinant Lm has great potential as a vaccine vehicle (10, 11, 25). Importantly for neonatal vaccination, recombinant attenuated strains of Lm induce Ag-specific immunity even in the presence of preexisting immunity (10), an issue of importance for vaccines where preexisting maternal immunity might interfere with the vaccine response in the newborn or infant. Based on our data, a single immunization with  $\Delta actA$ -Lm given at birth was sufficient to induce a long-lasting memory response. To our knowledge, this stands in sharp contrast to previously published records, where an adult booster dose was needed to maintain detectable levels of Ag-specific T cell memory (8). We thus reasoned that Lm could be an ideal vector for neonatal or infant vaccination. As a first test, we immunized neonatal and adult mice with  $\Delta actA$ -Lm-OVA and assayed the primary and secondary memory T cell response to OVA in parallel to the endogenous Lm Ags. We show in this study that after only one immunization, neonatally immunized mice generate OVA-specific primary and memory CD8 T cells with kinetics and at frequencies similar to adult-immunized mice, satisfying the requirement for a potential neonatal vaccine vehicle. To test this hypothesis directly, recombinant Ags from relevant pathogens expressed in  $\Delta actA$ -Lm would need to be tested in appropriate challenge models. This work is currently in progress.



**FIGURE 6.** Neonatally immunized mice develop an Lm- and OVA-specific secondary CD8 T cell response. Mice immunized with  $1 \times 10^4$  CFU i.p. of  $\Delta actA$ -Lm-OVA on day 5 of life (Neonate) or at 6 wk of age (Adult) were infected i.v. with  $1 \times 10^5$  CFU of WT Lm-OVA 3 mo after immunization. Spleen cells were obtained from the indicated mice 5 days after infection along with age-matched nonimmune (Naive) splenocytes and stimulated with the indicated MHC class I-restricted Lm- or OVA-specific peptides before analysis of intracellular cytokine expression by flow cytometry. **A**, Examples of the flow cytometric analysis. **B**, Mean percentage, and Total number per spleen of CD8 IFN- $\gamma$ -producing splenocytes (**C**). For each group, unstimulated IFN- $\gamma$ -producing controls are shown in the examples, as well as in the summary graphs (**B**), and the numbers of these cells were subtracted from the absolute numbers of Ag-specific cells shown. The data represent five mice per group from two combined experiments. Bar, SE.

The T lymphocyte response to pathogenic organisms focuses on a small number of epitopes, resulting in an epitope-specific CTL frequency hierarchy (i.e., immunodominance) (12, 26). We found that the fraction of the CD8 T cell response that was P60<sub>217-225</sub>- vs LLO<sub>91-99</sub>-specific was  $\sim 3$ -fold greater in mice immunized with  $\Delta actA$ -Lm as neonates compared with mice immunized as adults. And although LLO<sub>91-99</sub>-specific CD8 T cells increased to a dominant level in neonatally immunized mice during the secondary response, neonatally immunized mice still harbored  $\sim 3$ -fold more P60<sub>217-225</sub>-specific CD8 T cells than adults. This finding resembles what has been described in IFN- $\gamma$ -deficient adult mice (27). Neonates have been described to have reduced IFN- $\gamma$  production compared with adults (28, 29). IFN- $\gamma$  is known to induce the expression of molecules involved in MHC class I Ag processing and presentation (e.g., LMP2 and LMP7) in APCs, leading to a change from constitutive proteasomes to immunoproteasomes, which impacts Ag processing (30). Thus, reduced IFN- $\gamma$  production in neonates (and IFN- $\gamma$ -deficient adults) might prevent this switch, resulting in a P60-dominated response. Although the reasons for the difference in epitope hierarchy between the adult and neonatally immunized mice are presently not clear, the model described in this study should enable us to identify the mechanisms at the molecular level.

With the possibility to detect a significant primary and secondary immune response in neonatal mice using  $\Delta actA$ -Lm, we are now in a position to manipulate the factors determining the magnitude, tempo, and quality of the immune response and to delineate the mechanisms that lead to differences between the adult and neonate, such as epitope hierarchy. The induction in neonates of long-lived protective immunity without the need for a booster dose suggest  $\Delta actA$ -Lm, or derivatives thereof, as promising candidates for novel vaccine approaches in the neonatal setting.

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## Disclosures

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