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# Partial Activation of Neonatal CD11c$^+$ Dendritic Cells and Induction of Adult-Like CD8$^+$ Cytotoxic T Cell Responses by Synthetic Microspheres[1]

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**Neonatal cytotoxic T cell responses have only been elicited to date with immunogens or delivery systems inducing potent direct APC activation. To define the minimal activation requirements for the induction of neonatal CD8$^+$ cytotoxic responses, we used synthetic microspheres (MS) coated with a single CD8$^+$ T cell peptide from lymphocytic choriomeningitis virus (LCMV) or HIV-1. Unexpectedly, a single injection of peptide-conjugated MS without added adjuvant induced CD4-dependent Ag-specific neonatal murine cytotoxic responses with adult-like CTL precursor frequency, avidity for Ag, and frequency of IFN-γ-secreting CD8$^+$ splenocytes. Neonatal CD8$^+$ T cell responses to MS-LCMV were elicited within 2 wk of a single immunization and, upon challenge, provided similar protection from viral replication as adult CTLs, demonstrating their in vivo competence. As previously reported, peptide-coated MS elicited no detectable activation of adult CD11c$^+$ dendritic cells (DC). In contrast, CTL responses were associated with a partial activation of neonatal CD11c$^+$ DC, reflected by the up-regulation of CD80 and CD86 expression but no concurrent changes in MHC class II or CD40 expression. However, this partial activation of neonatal DC was not sufficient to circumvent the requirement for CD4$^+$ T cell help. The effective induction of neonatal CD8$^+$ T cell responses by this minimal Ag delivery system demonstrates that neonatal CD11c$^+$ DC may mature sufficiently to stimulate naive CD8$^+$ neonatal T cells, even in the absence of strong maturation signals.** ***The Journal of Immunology*****, 2004, 173: 2669–2674.**

The neonatal and early life period is characterized by an increased susceptibility to severe infections. This vulnerability has been attributed to the immaturity of the immune system, which includes limited capacities to rapidly elicit protective B and T cell responses (reviewed in Ref. 1). Neonatal T cell responses have demonstrated the preferential differentiation of CD4$^+$ T cells toward the Th2 pathway and the limited induction of CD8$^+$ cytotoxic responses, both in mice and in humans (2). These features were initially suggested as reflecting the existence of intrinsic neonatal T cell limitations (3, 4), although limitations of the function of APCs was suspected early (5). More recently, the induction of adult-like protective neonatal Th1 and CTL responses by Ag delivery systems such as DNA vaccines (6, 7), live replicating agents (8–11), or potent adjuvants (10, 12) known to upregulate APC function led to the hypothesis of a key role for neonatal APC activation in the shaping of T cell responses. This was further supported by reports showing that mimicking optimal APC activation by adoptive transfer of adult dendritic cells (DC)[4] (13), provision of exogenous IL-12 (10, 14–16), or direct triggering of CD40 via CD40L (17, 18) is sufficient to induce adult-like neonatal Th1 and CTL responses. Altogether, these observations supported a model in which the reduced ability of neonates to develop adult-like Th1 and CTL responses essentially reflects the immaturity of their DC, which appeared to require stronger and/or additional APC activation signals to efficiently prime and polarize naive CD8$^+$ and CD4$^+$ neonatal T cells. However, in contrast to these reports, peptide-pulsed neonatal CD11c$^+$ DC efficiently primed specific CTL responses when injected to adult mice (19) and nonreplicative virus-like particles elicited adult-like neonatal CD8$^+$ cell responses within 2 wk after priming, in absence of adjuvant (20).

The hypothesis of a limited activation capacity of neonatal murine DC was echoed by reports of limited IL-12 production and expression of costimulatory molecules by human neonatal monocyte-derived activated DC (21–24). Again, the provision of exogenous additional signals (IFN-γ) was sufficient to result in adult-like activation patterns of neonatal monocyte-derived DC, suggesting higher activation requirements of neonatal than of adult DC. Yet, the limited capacity for IL-12 production and expression of costimulation molecules did not prevent neonatal DC from successfully priming melanoma-specific human CTLs in vitro (25).

A consequence of these contrasting reports is that the minimal requirements for the efficient generation of CTL responses remain unknown, which limits the rational development of neonatal immunization strategies against intracellular pathogens for which the induction of cytotoxic responses would be desirable. To directly address the hypothesis that neonatal DC may be more dependent

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[4] Abbreviations used in this paper: DC, dendritic cell; MS, microsphere; LCMV, lymphocytic choriomeningitis virus; pf, precursor frequency.



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on strong APC activation signals than adult DC, we used a minimal Ag delivery system consisting of a single viral peptide conjugated to synthetic latex microspheres (MS; Ref. 26) to immunize 1-wk-old mice, whose immune maturation correlates most closely with that of human newborns (1). Unexpectedly, peptide-conjugated MS induce antiviral $CD8^+$ T cell neonatal responses that are adult-like by all aspects investigated. This activation of CTL responses is associated with a partial activation of neonatal $CD11c^+$ DC by these synthetic MS.

## Materials and Methods

### *Mice, virus, beads, and peptides*

BALB/c mice were purchased from IFFA CREDO (L'Abresle, France) and kept under specific pathogen-free conditions. Breeding cages were checked daily and the day of birth was recorded as the day the litter was found. Adult mice were used at 8–12 wk of age. Lymphocytic choriomeningitis virus (LCMV) strain, WE, was originally obtained as triple-plaque-purified stock from Dr. F. Lehmann-Grube (Heinrich-Pette Institut, Hamburg, Germany). Peptides carrying the p118–132 $CD8^+$ sequence from the LCMV strain WE nucleoprotein (27) or the p315–329 $CD8^+$ sequence from HIV-1 gp120 (28) were synthesized by Neosystem (Strasbourg, France). These peptides were covalently linked to the surface of 1-$\mu$m diameter synthetic latex particles (Polysciences, Warrington, PA) using glutaraldehyde as previously described (29). Similar MS labeled with the yellow-green fluorochrome (Polysciences) were used as tracers for in vivo uptake studies.

### *Immunization and in vivo depletion of $CD4^+$ T cells*

Mice were immunized i.p. in groups of 6–8 at 1 wk of age (neonatal immunization) or as adults (controls), with a single injection of $10^9$ MS-LCMV, MS-HIV, or MS-PBS, in the absence of any adjuvant. Where indicated, mice were injected i.p. with 300 $\mu$g of anti-CD4 (GK1.5) mAb prepared from ascitic fluid (30) on days −1, 0, +1, +7, and +11 of immunization. This resulted in greater than 90% $CD4^+$ T cell depletion in GK1.5-treated mice, as controlled by FACS analysis.

### *Quantification of cytotoxic responses and CTL precursor frequencies*

Equal numbers of splenocytes from individual mice were pooled 2 wk postimmunization and cultured as bulk or under limiting dilution conditions essentially as described (20). For bulk cultures, splenocytes were pooled by immunization groups and restimulated in vitro for 5 days with the $LCMV_{118–132}$ or the $HIV_{315–329}$ peptide, followed by a 4-h $^{51}$Cr-release assay with peptide-pulsed or unpulsed P815 target cells. For assessment of CTL avidity, dose-response curves were measured with titrated amounts of peptide pulsed on target cells, and the data was normalized to maximal response, according to the formula 100/(lysis at maximum peptide concentration − lysis without peptide) × (sample lysis − lysis without peptide). For limiting dilution analysis, titrated responder splenocytes were cultured for 10 days with $5 \times 10^5$ irradiated syngeneic stimulator spleen cells, peptide, and IL-2 as described (20), followed by a 4-h $^{51}$Cr-release assay of individual wells against peptide-pulsed P815 target cells.

### *ELISPOT assay of peptide-specific $CD8^+$ T cells producing IFN-$\gamma$*

The ELISPOT assay for detection of peptide-specific IFN-$\gamma$ secreting T cells was performed essentially as described (20). For analysis of ex vivo cytokine secretion, splenocytes were incubated for 48 h in ELISPOT plates with IL-2 and $10^6$ irradiated (3000 rad) syngeneic splenocytes in the presence or absence of specific peptide. For in vitro analysis, splenocytes from 5-day restimulated cultures used for cytotoxicity assay were cultured for 24 h in ELISPOT plates with IL-2 and peptide-pulsed or -unpulsed irradiated syngeneic splenocytes. Processing and counting were performed as previously described (20). The proportion of $CD8^+$ splenocytes was determined by flow cytometry before start of the incubation in ELISPOT plates.

### *Assessment of protective antiviral effect*

BALB/c mice were injected i.v. with an adult dose (200 PFU) of LCMV-WE, a nonlethal LCMV-WE strain, where protection is assessed by reduction of virus titers. Four or 5 days after challenge, as indicated, mice were sacrificed and virus titer determined in the spleen as described (20, 31). Virus titers are expressed as PFU per gram of spleen.

### *DC uptake and maturation analysis*

One-week-old BALB/c mice were injected i.p. with PBS or $5 \times 10^8$ fluorescent MS-LCMV MS (MS-YG-LCMV). Adult BALB/c mice were injected i.p. or i.v. (as indicated) with PBS or $10^9$ MS-YG-LCMV. Fifteen or 63 h later, splenocyte suspensions were incubated with MACS anti-CD11c beads (N418 clone, Miltenyi Biotec, Bergisch-Gladbach, Germany) and $CD11c^+$ cells were positively selected using high speed magnetic cell sorting (AutoMACS, Miltenyi Biotec). To analyze uptake, $CD11c^+$ were preincubated with rat anti-CD16/32 mAb (2.4G2 clone) and stained for 30 min with anti-CD11c-PE (HL-3 clone). For maturation analysis, incubation was performed with biotinylated anti-CD40 (3/23 clone), anti-CD80 (B7.1, 16-10A1 clone), -CD86 (B7.2, GL1 clone), -I-A/I-E (2G9 clone) mAbs and isotypes (BD Pharmingen, San Diego, CA). After washing and streptavidin-APC conjugated staining, propidium iodide was added to exclude dead cells. Each sample was acquired on the FACSCalibur cytometer and data were analyzed using the CellQuest Software (both BD Biosciences, San Diego, CA).

## Results

### *Synthetic MS induce adult-like in vitro cytotoxic responses in neonates*

BALB/c mice were immunized i.p. with a single dose of $10^9$ MS-LCMV or MS-PBS (control) at the age of 1 wk and as adult controls, in the absence of adjuvant. Two weeks after priming, splenocytes were restimulated in vitro with the $LCMV_{118–132}$ peptide and assessed for their capacity to lyse $LCMV_{118–132}$-pulsed P815 target cells. Immunization with MS-LCMV generated strong neonatal CTL responses: the restimulation of splenocytes from mice primed with MS-LCMV at 1 wk of age resulted in cytotoxicity against $LCMV_{118–132}$-coated target cells that was similar to that induced in adults, whereas no CTL responses were induced by control MS-PBS immunization (Fig. 1). It should be noted that CTL responses to $LCMV_{118–132}$ are not induced by immunization with free synthetic peptide alone (32, 33), such that these CTL responses are directly attributable to the use of MS as Ag delivery system. Considering the lower proportion of $CD8^+$ T cells in the spleen of neonates (which persists after in vitro restimulation, not shown), this indicated a significant cytotoxic potential of neonatal CTLs.

CTL responses were further characterized and quantified by limiting dilution analyses of spleens from neonatally primed mice, which detected high numbers of $LCMV_{118–132}$-reactive CTL precursors (Table I). CTL precursors were found at 2- to 3-fold lower

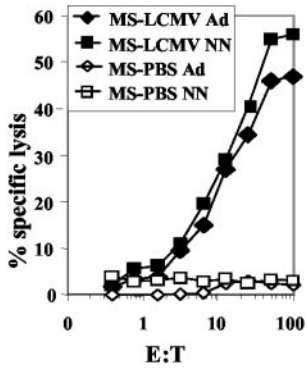


**FIGURE 1.** CTL responses to neonatal and adult MS-LCMV immunization. Neonatal (NN) and adult (Ad) BALB/c mice were immunized i.p. with $10^9$ MS-LCMV or MS-PBS. Two weeks after immunization, splenocytes were pooled by immunization groups and restimulated in vitro for 5 days with the $LCMV_{118–132}$ peptide. Cytotoxic activity was measured on $^{51}$Cr-labeled P815 targets cells pulsed with $LCMV_{118–132}$ or medium. Lysis of uncoated target cells by all effectors was <10% at all E:T ratios (not shown). Results are from one of three representative experiments.

Table I. *CTL frequencies after MS-LCMV immunization*

| Exp No. | Route | Age at Immunization | CTL pf | |
|---|---|---|---|---|
| | | | Per Splenocyte[a] | Per $CD8^+$ T Cell[b] |
| 1 | i.p. | Naive | 1/50,000 | 1/2,500 |
| | | Adults | 1/2,960 | 1/355 |
| | | Neonates | 1/5,113 | 1/257 |
| 2 | i.p. | Adults | 1/4,514 | 1/542 |
| | | Neonates | 1/9,686 | 1/484 |
| 3 | i.m. | Naive | 1/46,504 | 1/2,325 |
| | | Neonates | 1/7,896 | 1/394 |

[a] One-week-old and adult mice were immunized with MS-LCMV or left unprimed. CTL pf were assessed 2 wk later, after in vitro restimulation, as described in *Materials and Methods* CTL pf are expressed as the mean of the frequencies obtained in each immunization group.

[b] The frequency of LCMV-specific CTLs per $CD8^+$ T cells was calculated based on ex vivo flow cytometric enumeration of $CD8^+$ splenocytes.

numbers than in adults when expressed per total number of splenocytes. However, normalization to the frequency of $CD8^+$ cells present at culture initiation (3-wk-old, 5–7%; adult, 12–13% of total splenocytes) revealed an $LCMV_{118-132}$-reactive CTL precursor frequency (pf) at least as high in neonates as in adults. The high pf and the pf(adult):pf(neonatal) ratio did not depend on the route of immunization, because i.p. and i.m. administration yielded similar results (Table I). To define whether this unexpected induction of neonatal CTL by MS-LCMV reflected unique properties of the immunodominant LCMV CTL epitope used, similar experiments were performed with latex MS coated with the p315–329 peptide from HIV-1 gp120 (MS-HIV). Again, immunization induced a similar CTL pf in adults and neonates (1 of 11,810 splenocytes in neonates and 1 of 4,942 in adults in 1 representative of 2 experiments). Thus, adult-like cytotoxic antiviral responses are induced within 2 wk of neonatal priming with peptide-conjugated synthetic MS.

### *Neonatally MS-LCMV-induced CTLs have adult-like function and protective capacity*

We next asked whether the CTLs detected in mice primed as neonates were also able to secrete IFN-γ, the main effector CTL cytokine. ELISPOT analyses performed after in vitro restimulation detected similar frequencies of IFN-γ producers in neonatally or adult primed spleens (Fig. 2*A*). Similar frequencies of IFN-γ producers (1 of 243 splenocytes in neonates and 1 of 191 in adults, in 1 of 2 representative experiments) were obtained following immunization with MS-HIV. Importantly, IFN-γ production was not merely the result of prolonged in vitro restimulation, since low but reproducible numbers of IFN-γ-secreting cells were detectable ex vivo in response to short-term $LCMV_{118-132}$ stimulation (Fig. 2*B*). IFN-γ secretion was abrogated by complement-mediated depletion of $CD8^+$ cells, confirming $CD8^+$ splenocytes as the source of IFN-γ (not shown). Another important quality of antiviral CTL is their avidity for Ag, which correlates closely with protective efficacy against pathogenic challenge (34, 35). Neonatally induced anti-$LCMV_{118-132}$ CTL displayed an avidity profile identical with those induced in immunologically mature animals (Fig. 3*A*). To demonstrate that these responses are already elicited during the neonatal period, $CD8^+$ T cell responses were assessed 7 days after priming. At this early time point, LCMV-specific CTL responses were still heterogeneous, both in adults and in neonates. However, responding mice in both age groups had similar CTL and IFN-γ responses (not shown). Thus, CTL responses are elicited by peptide-coated MS in neonatal mice and they are equally efficient as those induced in adult mice.

Given the unexpected adult-like features of the neonatal CTL response generated by MS-LCMV, we analyzed their anti-viral protective activity in vivo. Neonates were primed with MS-LCMV and, to avoid induction of CTL at subsequent stages of greater immunological maturity, were challenged 2 wk later, at 3 wk of age. The MS-LCMV-induced CTL response resulted in a significant ($10^2$- to $10^3$-fold) reduction in splenic virus titers by day 4 after challenge, as compared with MS-PBS injected controls (Fig. 3*B*). This protection against viral replication was similar in mice immunized as neonates and as adults. When assessed 5 days after challenge, unprimed adult mice showed reduced viral titers (6.8 ± 0.14 log PFU/g), a consequence of the beginning primary antiviral CTL response. At this time point, viral titers were still high (7.75 ± 0.16 log PFU/g) in 3-wk-old MS-PBS control mice, indicating that the weaker clearance capacity of unvaccinated neonates extends to the age of 3 wk. In contrast, 40% of neonatally

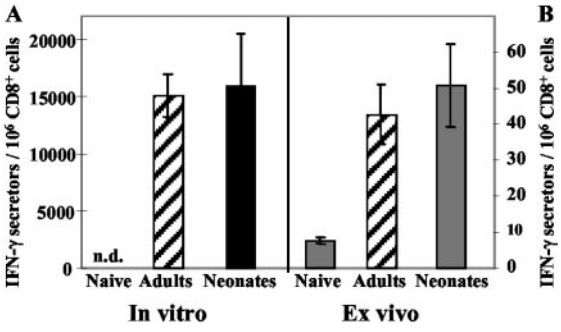


**FIGURE 2.** IFN-γ secreting $CD8^+$ splenocytes following neonatal and adult MS-LCMV immunization. Two weeks after MS-LCMV immunization of BALB/c mice, splenocytes were restimulated in vitro with $LCMV_{118-132}$ during 5 days (*A*) or used ex vivo (*B*). The frequency of IFN-γ secreting cells was assessed in individual mice by ELISPOT, as described in *Materials and Methods*. Data are expressed as the number of spots-forming cells per million $CD8^+$ T cells, as determined by flow cytometry before start of the assay, after subtraction of spots-forming cells in wells without $LCMV_{118-132}$ (always <5). Error bars depict SD from six to eight mice. Results are representative of three experiments.

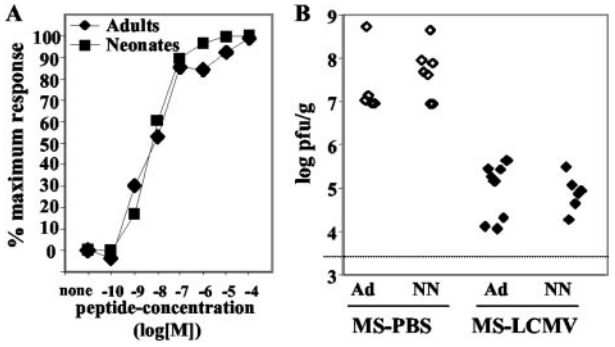


**FIGURE 3.** Ag avidity and protective efficacy of MS-LCMV-induced responses. *A*, Two weeks after MS-LCMV immunization of BALB/c mice, splenocytes were pooled by immunization group and restimulated in vitro with $LCMV_{118-132}$ before evaluation of cytotoxicity on $^{51}Cr$-labeled P815 targets cells pulsed with various concentrations of $LCMV_{118-132}$ peptide. Data represent the means of the percent of specific lysis from duplicate wells obtained at a 60:1 E:T ratio. Results are representative of two experiments. *B*, Two weeks after MS-LCMV or MS-PBS immunization, adult (Ad) or neonatal (NN) mice were injected i.v. with 200 PFU of LCMV-WE and viral titers were assessed in the spleen 4 days later, as described in *Materials and Methods*. Virus titers are expressed as PFU per gram of spleen tissue. Each diamond represents an individual mouse. The dotted line represents the assay cutoff. Results are from one representative of three experiments.

MS-LCMV-primed mice had no detectable virus 5 days after challenge. Thus, neonatal immunization with synthetic MS conjugated with viral peptides elicits fully competent effector CTL that have adult-like function and protective capacity against viral replication.

*Activation of neonatal DC by synthetic MS*

The capacity of latex MS to induce adult-like CTL responses prompted us to assess whether this was associated with direct uptake and activation of neonatal DC, the only APC capable to stimulate naive T cells. Fluorescent MS carrying the LCMV $NP_{118-132}$ $CD8^+$ T cell determinant (MS-YG-LCMV) were injected i.p. into 1-wk-old BALB/c mice (or i.v. to adult mice) before isolation and analysis of splenic $CD11c^+$ DC by flow cytometry. Synthetic MS are taken up by a proportion (1.2–2.4%) of neonatal $CD11c^+$ DC that is similar to that observed following adult immunization (1.7–1.9%; Fig. 4). In adult mice, the in vivo uptake of MS-YG-LCMV by $CD11c^+$ DC did not induce any detectable up-regulation of CD40, CD80, CD86, or MHC class II (Fig. 4, *A* and *C*), as previously reported in vitro (36). Unexpectedly, the expression of CD80 and CD86 was increased on a large fraction (38–62%) of neonatal MS-YG-$LCMV^+$ DC. This activation was transient, because it was observed 15 h (Fig. 4*B*) but not 63 h (Fig. 4*D*) after administration, and was limited to MS-YG-$LCMV^+$ DC, i.e., not detected by the analysis of the entire $CD11c^+$ DC population (not shown). It was not associated with changes in the expression of CD40 and MHC class II expression (Fig. 4, *A* and *B*). The lack of activation of adult MS-YG-$LCMV^+$ DC did not reflect the use of the i.v. injection route: the i.p. injection of MS-YG-LCMV proved less efficient (0.1% MS-YG-$LCMV^+$ DC) and did not induce any detectable activation of $MS^+CD11c^+$ DC (not shown). Thus, the injection of adjuvant-free synthetic MS resulted in their uptake by neonatal as well as by adult $CD11c^+$ DC. This was sufficient to enhance the expression of the CD80/CD86 costimulation molecules on neonatal but not on adult $MS^+CD11c^+$ DC.

Optimal CTL-Th cell interactions are considered essential for the induction of fully competent CTL responses (37, 38). However, Ag delivery systems capable of inducing direct in vivo APC activation may not require $CD4^+$ T cell help (reviewed in Ref. 39). Given the capacity of MS to induce the up-regulation of CD80/CD86, we asked whether this would be sufficient for MS-LCMV immunization to circumvent the requirement for $CD4^+$ T cell help in the induction of neonatal LCMV-specific $CD8^+$ T cells. However, the depletion of $CD4^+$ cells abolished the cytotoxic response induced by MS-LCMV in neonates (Fig. 5), as previously reported in adult mice (26). Therefore, $CD4^+$ T cell help remains essential for the induction of neonatal LCMV-specific $CD8^+$ T cells by MS-LCMV. Remarkably, this $CD4^+$ T cell help occurs despite the fact that the putative MHC class II-restricted LCMV epitope contained within $LCMV_{118-132}$ is too weak to permit detection of LCMV-specific cytokine (IFN-γ, IL-5, and IL-4) responses following in vitro restimulation of splenocytes from mice immunized either as neonates or as adults (not shown).

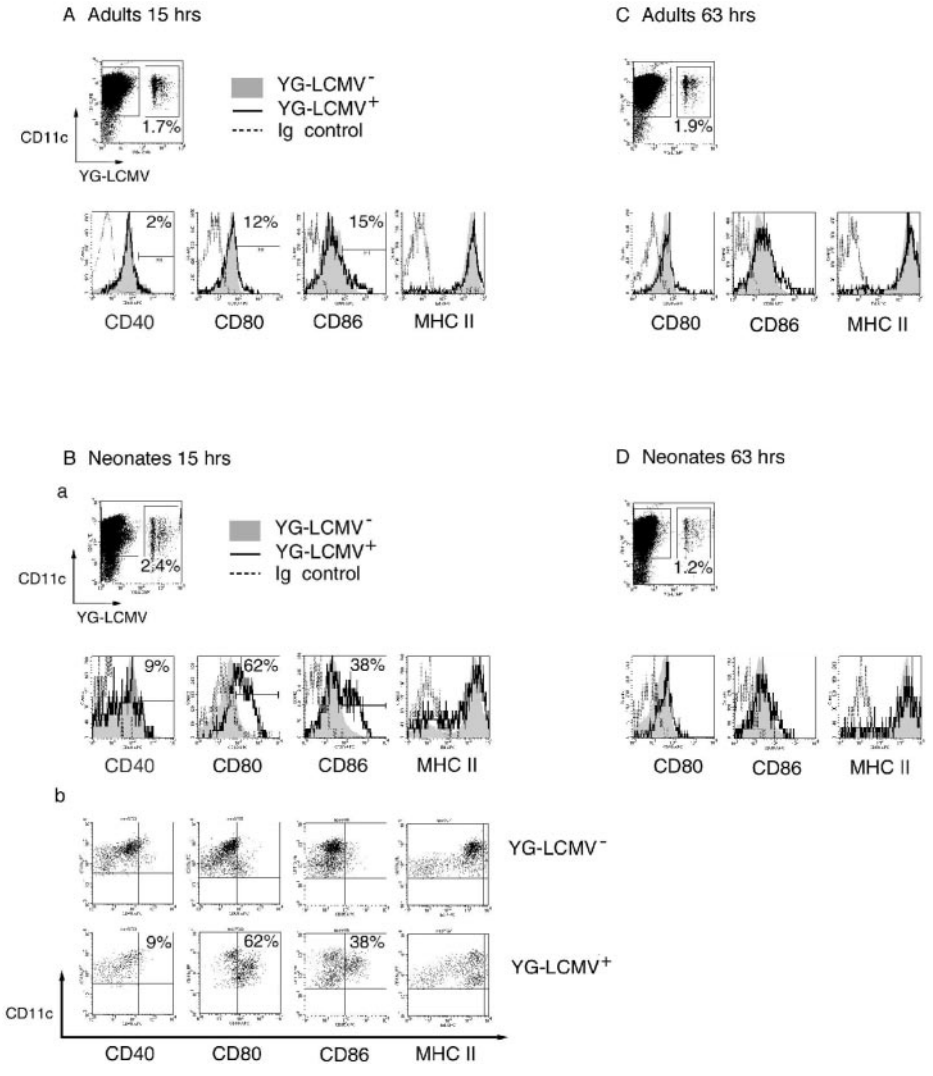


**FIGURE 4.** Influence of MS-YG-LCMV on DC maturation. MS-YG-LCMV were injected i.v. to adult (*A* and *C*) and i.p. to 1-wk-old (*B* and *D*) BALB/c mice. Fifteen (*A* and *B*) or 63 h (*C* and *D*) later, splenic DCs were purified and stained with anti-CD11c PE mAb in combination with anti-CD40, CD80, CD86, and $I\text{-}E^d$ biotin mAbs followed by SA-APC staining. In *A* and *Ba*, *C* and *D*, dot plots show the gates of $CD11c^+$/YG-$LCMV^-$ and $CD11c^+$/YG-$LCMV^+$ DCs and histograms show the maturation state of $CD11c^+$/YG-$LCMV^-$ DCs (gray histogram), $CD11c^+$/YG-$LCMV^+$ (bold line) and isotype control staining (dotted line). *Bb* shows the comparison of DC maturation in $CD11c^+$/YG-$LCMV^+$ and $CD11c^+$/YG-$LCMV^-$ DC. Dot plots show total $CD11c^+$/YG-$LCMV^-$ (*upper panels*) and $CD11c^+$/YG-$LCMV^+$ gated DC (*lower panels*). Results are representative of one of three independent experiments.

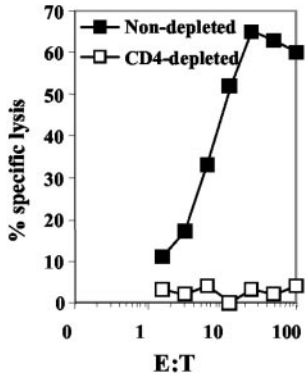


**FIGURE 5.** Role for $CD4^+$ T cells in induction of CTL responses by MS-LCMV. Neonates were injected i.p. with 300 $\mu$g of anti-CD4 (GK1.5) mAb, as described in *Materials and Methods*, before MS-LCMV immunization. Two weeks after immunization, splenocytes were pooled by immunization groups and restimulated in vitro for 5 days with the $LCMV_{118-132}$ peptide. Cytotoxic activity was measured on $^{51}Cr$-labeled P815 targets cells pulsed with $LCMV_{118-132}$ or medium. Lysis of uncoated target cells by all effectors was <10% at all E:T ratios (not shown). Results are from one of three representative experiments.

## Discussion

We demonstrate in this study that a minimal Ag delivery system consisting of a single viral peptide conjugated to latex MS is sufficient to partly activate neonatal $CD11c^+$ DC, and that this minimal DC activation is sufficient for the induction of adult-like protective neonatal $CD8^+$ T cell responses.

Whether the limitation of the induction of $CD8^+$ T cell neonatal responses essentially results from limitations of neonatal DC, $CD4^+$, and/or $CD8^+$ T cells is an important and ongoing debate. To directly address the hypothesis that neonatal DC may be more dependent on strong APC activation signals than adult DC, we have used a minimal Ag delivery system consisting of a single viral peptide conjugated to synthetic latex MS. We show here that these MS are as readily taken up by neonatal as by adult $CD11c^+$ DC, despite the lack of added pathogen-associated molecular patterns or DC targeting signals. This is in accordance with previous reports showing the capacity of splenic DC to take up latex MS in vivo (40) and with the localization of these particles in DC and marginal zone macrophages following injection (41).

Despite their targeting capacities, latex MS do not induce detectable activation of adult $MS^+CD11c^+$ DC in vitro (36) or in vivo (our results), in contrast to added pathogen-associated molecular patterns-containing molecules. The first unexpected finding is thus that following injection into a neonatal environment, the in vivo uptake of peptide-conjugated MS results in the partial activation of neonatal $CD11c^+$ DC. The up-regulation of CD80 and CD86 expression was not identified when studying the whole $CD11c^+$ DC population, highlighting the importance of assessing MS-targeted DC. However, this is not trivial when experimental conditions result in few cells being amenable for analysis (700–2400 per spleen; Fig. 4), as in neonatal immunization models. This up-regulation of CD80/CD86 was repeatedly observed on a large fraction (40–60%) of $MS^+CD11c^+$ DC, indicating that it is not a marginal event. It occurred early, with detection on splenic $CD11c^+$ DC as early as 15 h after immunization suggesting direct DC activation, and was transient, as analyses performed 5 days after priming indicated that CD80 and CD86 expression levels had returned to baseline in $MS^+CD11c^+$ DC. This activation process was partial, as it was not associated with enhanced expression of MHC class II molecules or of CD40, which was only expressed on 9% of $MS^+CD11c^+$ neonatal DC. Although we cannot formally exclude that the lack of activation observed in adult DC reflects a difference between baseline adult and neonatal DC CD80/CD86 expression levels (Fig. 4), this is not systematically observed and rather suggests intrinsic characteristics of the neonatal immune system. Detailed analyses of the functional capacity of MS-induced neonatal DC and of the mechanisms allowing their partial activation is unfortunately limited by the low number of $CD11c^+$ DC cells in the spleen of 1-wk-old mice ($1.1–2.3 \times 10^5$; Ref. 42). Nonetheless, we can conclude that murine neonatal $CD11c^+$ DC may be activated at least to some extent by Ag delivery systems that do not include known activators capable to mediate potent APC activation.

The second unexpected finding was that this partial activation of neonatal $CD11c^+$ DC was associated with $CD8^+$ T cell responses with adult-like characteristics: the frequency of CTL precursors, the cytotoxic capacity of $CD8^+$ T cells, their IFN-$\gamma$ production, and the protective capacity against viral replication was similar in mice immunized as neonates or as adults. These responses were elicited within 1–2 wk after priming, i.e., during the neonatal period. We do not conclude from these observations that synthetic MS induce optimal CTL responses, and we recently reported significantly stronger CTL responses and immunity against LCMV challenge in neonatal mice immunized with virus-like particles encoding for the same $LCMV_{118-132}$ determinant as the peptide-conjugated MS used in this report (20). Rather, the conclusion from our observations is that synthetic latex MS are able to generate similar $CD8^+$ T cell responses capable of similar viral clearance following neonatal and adult immunization. Thus, neonatal murine $CD11c^+$ DC do not exhibit stronger activation requirements for $CD8^+$ T cells than their adult counterparts, and neonatal $CD8^+$ T cells do not have intrinsic deficiencies in terms of T cell avidity and ability to develop effector cytotoxic functions on a per $CD8^+$ T cell basis.

The partial activation of neonatal DC by peptide-conjugated MS was not sufficient to circumvent the requirement for $CD4^+$ T cell help following MS immunization, either in neonates (this report) or in adults (26). Therefore, this CD4-dependence does not represent a hallmark of neonatal DC activation capacity. How $CD4^+$ T cells provide the required help in this model is an interesting question. We did not detect any of the typical Th cytokines following in vitro restimulation with the $LCMV_{118-132}$ peptide (not shown), although local in vivo production cannot be excluded (29, 43). The lack of induction of CD40 on $CD11c^+$ $MS^+$DC suggests to us that, in this model, the $CD4^+$ T cell help to neonatal $CD8^+$ T cells could be essentially mediated by direct T-T collaboration via recently described CD40/CD40L interactions (37). This is supported by our unpublished observations that anti-CD3 activated $CD4^+$ T cells of 1-wk-old mice express adult levels of costimulation molecules, including CD40L, Ox40, and CD137/4–1BB, in contrast to reports of limited CD40L expression on activated $CD4^+$ T cells from 2-day-old mice (18). Of course, we cannot exclude that $CD4^+$ T cell help to MS-induced $CD8^+$ T cells could be mediated by still undefined CD40-independent pathways (44). However, MS conjugated to an OVA CTL epitope (aa 257–264) known not to contain a Th epitope failed to elicit $CD8^+$ T cell responses unless additional activation was provided by anti-CD40 stimulation (F. Boisgérault, P. Rueda, C.-M. Sun, M. Rojas, and C. Leclerc, manuscript in preparation). This further supports the role of $CD4^+$ T cells in the induction of $CD8^+$ responses to MS. That neonatal $CD4^+$ T cells may be sufficiently activated by an Ag delivery system as minimal as latex MS to provide efficient help is also unexpected in view of the reported limitations of neonatal $CD4^+$ T cells (2) and deserves further evaluation. It suggests that the

most frequently assessed functional properties of $CD4^+$ T cells, cytokine production and proliferation, may fail to reveal important aspects of neonatal Th function.

This is, to our knowledge, the first report demonstrating that a minimal Ag delivery system based on latex MS is capable of potent and rapid induction of adult-like CTLs in early life. The observation that 1) neonatal murine $CD11c^+$ DC do not require strong activation to initiate $CD8^+$ T cell priming (Refs. 19 and 42 and this report), 2) neonatal $CD8^+$ T cells do not have intrinsic deficiencies in terms of T cell avidity and ability to develop effector cytotoxic functions on a per $CD8^+$ cell basis (Refs. 7 and 20 and this report), and 3) neonatal $CD4^+$ T cell responses without detectable cytokine secretion provide significant help for the induction of adult-like protective CTL responses (this report) indicate that other factors influence the APC-$CD8^+$ T cell interactions when more complex Ag delivery systems are used in a neonatal microenvironment. We are currently testing the hypothesis that the limitations of neonatal CTL responses observed in many experimental systems may result from the induction of inhibitory mechanisms triggered under most—but not all—experimental conditions. Regardless of the exact mechanisms, the demonstration that a minimal Ag delivery system is capable of inducing adult-like neonatal $CD8^+$ T cell antiviral responses opens new perspectives for immunization strategies that should elicit protective cytotoxic responses already in neonates.

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