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Efficient In Vivo Priming of Specific Cytotoxic T Cell Responses by Neonatal Dendritic Cells[1]

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In early life, a high susceptibility to infectious diseases as well as a poor capacity to respond to vaccines are generally observed as compared with observations in adults. The mechanisms underlying immune immaturity have not been fully elucidated and could be due to the immaturity of the T/B cell responses and/or to a defect in the nature and quality of Ag presentation by the APC. This prompted us to phenotypically and functionally characterize early life murine dendritic cells (DC) purified from spleens of 7-day-old mice. We showed that neonatal CD11c$^+$ DC express levels of costimulatory molecules and MHC molecules similar to those of adult DC and are able to fully maturate after LPS activation. Furthermore, we demonstrated that neonatal DC can efficiently take up, process, and present Ag to T cells in vitro and induce specific CTL responses in vivo. Although a reduced number of these cells was observed in the spleen of neonatal mice as compared with adults, this study clearly shows that neonatal DC have full functional capacity and may well prime Ag-specific naive T cells in vivo. ***The Journal of Immunology,*** **2002, 168: 2219–2224.**

The neonatal period is marked by a high susceptibility to infections. This susceptibility is associated with a marked limitation of the capacity of most vaccines to induce protective immune responses, as observed in neonates and young infants as well as in newborns from various animal species including mice (1). The understanding of immune immaturity is an important issue given the global burden of infectious diseases in early life. However, the mechanisms responsible for the limitations of infant immune responses are not yet fully elucidated. Although studies of neonatal T lymphocyte function showed several intrinsic defects such as low proliferation rate in response to anti-CD3 stimuli (2), Th2-biased response (3, 4), or weak expression of CD40 ligand on activation (5), recent findings suggest that this could be essentially attributed to the nature and quality of Ag presentation by professional cells, as demonstrated previously for neonatal macrophages (6). However, it is now well established that dendritic cells (DC)[3] are the main APC with a unique ability to sensitize naive T cells and induce primary immune responses (7). Human cord blood DC were recently shown to be less efficient than adult DC in supporting proliferation of T cells in response to antigenic stimulation (8). Because DC drive the development of Th1 response through the production of IL-12, which appears limited both in murine and human neonates, it was also suggested that the lack of Th1 response observed in neonatal mice could be due to suboptimal Th1 cell activation by neonatal DC (1). Recently, adherent cells (including DC) from spleens of mice <4 wk of age were shown to be defective in presenting tetanus toxoid to Ag-specific T cell clones (9). Altogether, these observations suggested that neonate DC may not be fully functional and could present a limited efficiency to induce Th1 and CTL activation. This prompted us to phenotypically and functionally characterize early life murine DC, including their ability to specifically activate CD8$^+$ T cells in vivo. In this study, DC were purified from spleens of 7-day-old mice, which best corresponds to the stage of immune maturation of human newborns in terms of vaccine Ab responses and maturation of T cell responses (reviewed in Ref. 1). The number and the phenotype of these cells were characterized, and the function of these neonate DC was analyzed by determining their ability to mature, take up, process, and present Ag to CD8$^+$ T cells, as well as their capacity to induce in vivo specific CTL responses.

Materials and Methods

Mice

BALB/c mice, originally obtained from CER Janvier (Le Genest St. Isle, France), were bred and housed onsite. Periodic screening showed the colony to be free of commonly occurring infectious agents. Breeding cages were checked daily for new births, and the day of birth was recorded as the day the litter was found. Pups were weaned, and males and females were separated at 3 wk of age. Neonatal animals were defined as those 7 days old, and adult mice used in this study were 8–10 wk old.

Peptide and detoxified recombinant adenylate cyclase

The synthetic peptide p118–126 (RPQASGVYM) carrying the 118–126 sequence from the lymphocytic choriomeningitis virus (LCMV) nucleoprotein corresponding to a class I and class II H-2^d-restricted epitope (10, 11) was purchased from Neosystem (Strasbourg, France). This peptide was tested for the presence of endotoxin using a chromogenic test (*Limulus* amebocyte lysate test; BioWhittaker, Fontenay-sous-Bois, France), and no endotoxin was detected.

The detoxified adenylate cyclase from *Bordetella pertussis* (CyaA-E5) and the CyaA-E5 bearing the LCMV epitope (CyaA-E5-LCMV) were gifts from D. Ladant (Pasteur Institute, Paris, France). Their production and purification have been previously described (12).

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[3] Abbreviations used in this paper: DC, dendritic cells; LCMV, lymphocytic choriomeningitis virus; CyaA-E5, detoxified adenylate cyclase from *Bordetella pertussis*. CM, complete medium.

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Culture medium and cell line

Complete medium (CM) consisted of RPMI 1640 containing L-alanyl-L-glutamine dipeptide supplemented with 10% FCS, 5×10^{-5} M 2-ME, and antibiotics (penicillin, 100 U/ml, and streptomycin, 100 μg/ml).

The MV1H7 T cell hybridoma specific for the H-2^d-restricted 118–126 LCMV epitope was generated in the laboratory as previously described (13).

DC purification

DC were purified from spleens of 7-day-old or 8- to 10-wk-old mice by positive selection according to CD11c expression using Automacs (Miltenyi Biotec, Bergisch Gladbach, Germany), following the manufacturer's procedure. The purity of the two fractions was checked by flow cytometry on a FACSCalibur or FACScan (BD Biosciences, San Jose, CA), using a PE-conjugated anti-CD11c (HL3 clone; BD PharMingen, San Diego, CA) for labeling. The purity of the fraction was usually >97%.

Flow cytometry analysis

Cells were washed in PBS supplemented with 1% BSA and 0.1% NaN_3, incubated with anti-CD32/CD16 (2.4G2 clone; BD PharMingen) to avoid nonspecific staining, and labeled for 30 min at 4°C with the following Abs purchased from PharMingen: PE- or APC-conjugated anti-CD11c mAb (HL3 clone); PerCP-conjugated anti-CD3 mAb (17A2); PE-conjugated anti-CD4 mAb (RM4-5); FITC-conjugated anti-CD8 (53-6.7); PE-conjugated anti-CD45R/B220 mAb (RA3-6B2); FITC-conjugated anti-CD40 mAb (3/23); FITC-conjugated anti-CD80 mAb (16-10A1); FITC-conjugated anti-CD86 mAb (GL1), FITC-conjugated anti-I-A^d mAb (AMS-32.1). As negative controls, cells were stained with corresponding isotype-matched control mAbs. Acquisition was performed on a FACSCalibur flow cytometer, and analysis was done with CellQuest (BD Biosciences).

LPS maturation

Purified DC from spleens of 7-day-old and 8-wk-old BALB/c mice were cultured in CM containing 10 μg/ml LPS from *Escherichia coli* (Sigma-Aldrich, Steinheim, Germany) during 6 h. Expression of maturation markers CD40, CD80, CD86, and MHC class II molecules was assessed by flow cytometry analysis before and after LPS stimulation. DC maturation was also assessed by the lost of endocytic capacity (see below).

Ag uptake assay

Freshly purified DC (immature DC) from spleens of 7-day-old and 8-wk-old BALB/c mice were incubated 1 h at 4°C or 37°C in CM containing 1 mg/ml FITC-dextran (40 kDa; Sigma-Aldrich). After two washes in PBS supplemented with 1% BSA and 0.1% NaN_3, cells were stained with PE-conjugated anti-CD11c mAb, and then FITC-dextran endocytosis by DC was assessed by flow cytometry analysis on gated $CD11c^+$ cells. The same experiment was also performed on purified DC after overnight culture in the presence of 10 μg/ml LPS (mature DC).

Peptide presentation to T cell hybridoma

The stimulation of T cell hybridoma was monitored by IL-2 release in the culture supernatants in the presence of $CD11c^+$ DC from spleens of 7-day-old or 8- to 10-wk-old BALB/c mice. Purified $CD11c^+$ DC (2×10^4) were incubated with various Ag concentrations during 4 h at 37°C. In some experiments, various numbers of DC were incubated with a fixed concentration of Ag. After washing, 5×10^4 T cell hybridoma cells were cocultured in 96-well culture plates in the presence of DC. After 18 h, cultures were centrifuged, and cell-free supernatants were frozen for at least 2 h at −80°C. Then, 10^4 cells/well of the IL-2 dependent CTLL-2 cell line were cultured with 100 μl of these supernatants. After 48 h, [^{3}H]thymidine (50 μCi/ml; ICN, Orsay, France) was added in the wells, and the cells were harvested 6 h later with an automated cell harvester (Skatron, Lier, Norway). Incorporated thymidine was detected by scintillation counting. In all experiment, each point was done in duplicate. Results are expressed as the mean cpm.

MLR assay

$CD8^+$ T cells were purified from spleens of adult BALB/c or C57BL/6 mice by positive selection according to CD8α expression using Automacs (Miltenyi Biotec), following the manufacturer's procedure. Then, 1×10^5 cells were cocultured with various numbers of $CD11c^+$ cells purified from spleens of 7-day-old or 8-wk-old BALB/c mice. After 72 h, [^{3}H]thymidine (50 μCi/ml; ICN) was added to the wells, and the cells were harvested 6 h later with an automated cell harvester (Skatron). Incorporated thymidine was detected by scintillation counting. In all experiments, each point was done in duplicate. Results are expressed as the mean cpm.

Mouse immunization

Purified $CD11c^+$ dendritic cells from spleens of 7-day-old or 8- 10-wk-old BALB/c mice were incubated for 1 h with or without 50 μM $p_{118-126}$ in Sfem Stem Span medium (StemCell, Meylan, France). After extensive washing, 2×10^5 cells were i.v. injected into 8- to 10-wk-old syngeneic mice.

In vitro cytotoxicity assay

Splenocytes from immunized mice were isolated 7 days after DC injection and restimulated in vitro during 5 days with $p_{118-126}$ (0.1 μg/ml) in the presence of syngeneic irradiated naive spleen cells. The cytotoxic activity was determined in a 5-h in vitro ^{51}Cr release assay as previously described (12). Briefly, P815 tumor cells incubated with or without the $p_{118-126}$ peptide (50 μM) were used as target cells. Various E:T ratios were used, and all assays were performed in duplicate. ^{51}Cr release in each well was counted using a MicroBeta Trilux liquid scintillation counter (Wallac, Turku, Finland). Percentage of specific lysis was calculated as 100 × [(experimental release − spontaneous release)/(maximal release − spontaneous release)]. Maximum release was obtained by adding 10% Triton X-100 to target cells, and spontaneous release was determined with target cells incubated without effector cells. Results are expressed as Δ% of specific lysis (% of lysis in the presence of peptide − % lysis in the absence of peptide).

Results

Number and phenotypical characterization of neonatal DC

It is well established that functional splenic DC in adult mice express high levels of the CD11c marker. Thus, to analyze the function of neonatal DC, $CD11c^+$ splenocytes were isolated by positive selection on MACS from spleens of 7-day-old BALB/c mice using magnetic CD11c microbeads. The number of cells recovered was compared with the number of DC isolated from adult BALB/c mice (Table I); 12- to 50-fold less $CD11c^+$ cells were found into the spleen of 7-day-old mice as compared with spleen from adult mice. Indeed, $0.1–0.4 \times 10^6$ $CD11c^+$ cells were recovered in their spleens, corresponding to 0.15–0.7% of splenocytes, whereas 3.3% of cells expressed the CD11c molecule in the spleen of adult mice. However, DC:T cell and DC:B cell ratios in the spleens of 7-day-old and adult mice were quite similar. Expression of costimulatory molecules and MHC molecules was also assessed on neonatal $CD11c^+$ cells and was compared with the expression of these markers on adult DC (Fig. 1). Both neonatal and adult DC expressed similar levels of CD40, CD80, CD86, and MHC class II

Table I. *Number and percentage of $CD11c^+$ cells in spleens from 7-day-old and 8-wk-old BALB/C mice*[a]

Spleen	Total No. of Splenocytes ($\times 10^6$)	No. of $CD11c^+$ Cells ($\times 10^6$)	% of $CD11c^+$ Cells	DC:T Cell Ratio	DC:B Cell Ratio
7-day-old mice	55–75	0.1–0.4	0.15–0.7	0.1	0.028
8-wk-old mice	150	5	3.3	0.08	0.04

[a] Number and percentage of $CD11c^+$ cells in spleen were determined by flow cytometry. Data represent results obtained from 10 mice. DC:T and DC:B cell ratios were determined after flow cytometry analysis; DC are characterized as $CD11c^+$ cells, T cells are characterized as $CD3^+$ and $CD8^+$ or $CD4^+$, and B cells are characterized as $B220^+$ cells. Data represent results obtained from five mice.

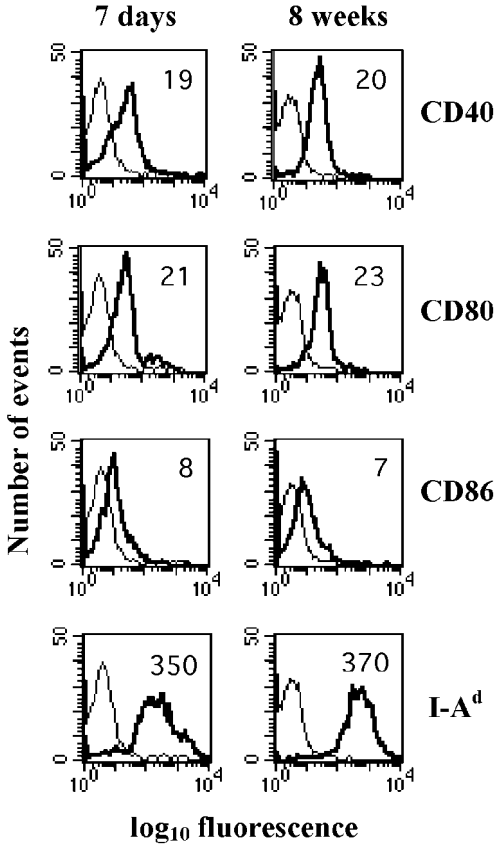

FIGURE 1. Phenotypical analysis of neonatal and adult DC. CD11c$^+$ cells were isolated from spleens of 7-day-old (neonate) and 8-wk-old (adult) BALB/c mice and double-stained with PE-labeled CD11c and FITC-labeled anti-CD40, anti-CD80, anti-CD86, anti-I-A^d, or isotype control, respectively. Histograms show the CD40, CD80, CD86, and I-A^d expression profiles (bold histograms) and isotype control (thin histograms) on gated CD11c$^+$ cells. The median of fluorescence intensity for all markers is indicated in each quadrant.

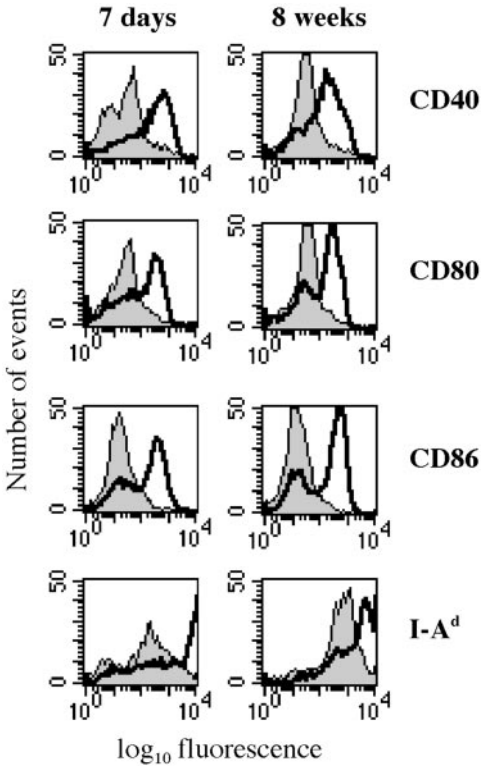

FIGURE 2. Neonatal DC up-regulate maturation markers after LPS stimulation. CD11c$^+$ cells were isolated from spleens of 7-day-old (neonate) and 8-wk-old (adult) BALB/c mice and double-stained with PE-labeled CD11c and FITC-labeled anti-CD40, anti-CD80, anti-CD86, and anti I-A^d before and after 6 h of in vitro stimulation with 10 μg/ml LPS. Histograms show the CD40, CD80, CD86, and I-A^d expression profiles before (gray histograms) and after (white histograms) 6 h in vitro LPS stimulation.

molecules, indicating that expression of the main molecules involved in DC-T cell interaction at the surface of neonatal CD11c$^+$ DC was not affected.

Ability of neonatal DC to mature following LPS stimulation

DC represent a heterogeneous population of cells. Immature DC are characterized by an efficient capacity to capture Ag but a low efficiency to stimulate naive T cells. After Ag capture and activation by microbial or inflammatory stimuli, DC mature and acquire the ability to stimulate naive T cells. DC maturation is characterized by up-regulation of expression of costimulatory and MHC molecules at the surface of DC (7). Thus, to determine the ability of neonatal DC to mature, purified CD11c$^+$ splenocytes from 7-day-old BALB/c mice were stimulated 6 h in vitro with LPS. The level of expression of CD40, CD80, CD86, and I-A^b molecules was determined by FACS analysis and compared with the expression level of these markers before stimulation. Fig. 2 shows that LPS induced a marked increase in the surface expression of CD40, CD80, and CD86 markers with the same efficiency than adult CD11c$^+$ cells. Furthermore, a high increase of MHC class II expression was observed for both neonatal and adult CD11c$^+$ cells after LPS stimulation although this increase cannot be compared between these two populations due to very high levels of expression. Altogether, these results clearly show the full maturation potential of neonatal DC.

Ag uptake by neonatal DC

Immature DC are very efficient in Ag capture using several pathways and DC maturation process is associated with a loss of endocytic and phagocytic capacities (7). To compare the Ag uptake capacity between neonatal and adult DC, the endocytic activity of these cells was assessed by determining the internalization of FITC-conjugated dextran by immature and mature purified DC from spleens of 7-day-old and 8-wk-old BALB/c mice. Freshly purified DC were incubated for 1 h at 37°C with FITC-dextran; then its internalization was assessed by flow cytometry on gated CD11c$^+$ cells. As controls, purified DC were incubated at 4°C to block endocytosis (Fig. 3). Immature neonatal and adult DC show similar efficacy in the uptake of FITC-dextran, demonstrating the efficient endocytic capacity of neonatal DC. As expected, after maturation following LPS stimulation, both neonatal and adult DC lose their endocytic capacity as shown by the similar profile obtained after incubation at 4°C and 37°C for both DC populations. These results clearly show the capacity of neonate DC to take up Ag and confirm the ability of these cells to mature after LPS stimulation.

Ag processing and presentation by neonatal DC

The ability of neonatal DC to present Ag was assessed by their capacity to stimulate the MV1H7 CD8$^+$ T cell hybridoma, specific for the L^d-restricted epitope 118–126 from the nucleoprotein of LCMV. Purified CD11c$^+$ splenocytes from 7-day-old BALB/c mice were loaded with various concentrations of the $p_{118-126}$ synthetic peptide. These cells were used as APC to stimulate the

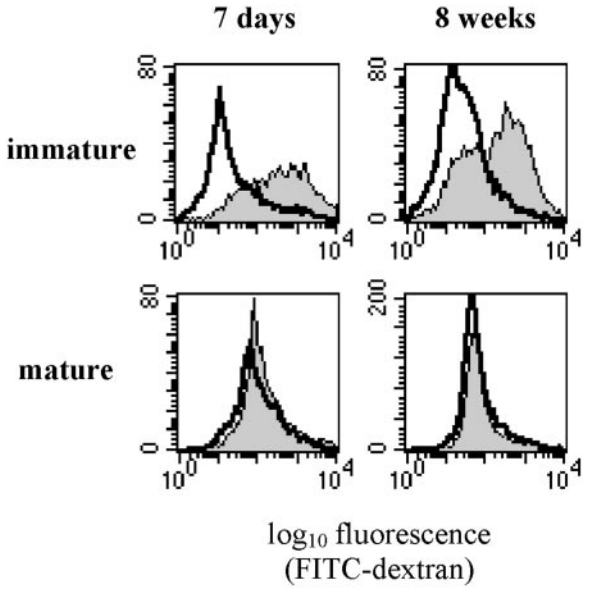

FIGURE 3. Efficient Ag uptake by neonatal and adult DC. *Top row*, Freshly purified CD11c$^+$ cells (immature DC) from spleen of 7-day-old (neonate) and 8-wk-old (adult) BALB/c mice were incubated in the presence of FITC-dextran at 37°C (gray histograms) or 4°C (white histograms) during 1 h. Then, cells were stained with PE-labeled anti-CD11c before FACS acquisition. Histograms show the uptake of FITC-dextran on gated CD11c$^+$ cells. *Bottom row*, The same experiment was performed using purified CD11c$^+$ cells after overnight culture in the presence of LPS (mature DC). Results are representative of two experiments.

MV1H7 hybridoma, and the amount of IL-2 secreted into culture supernatant was determined (Fig. 4*A*). Neonatal DC stimulated this MHC class I-restricted hybridoma with the same efficiency as that of adult DC because both DC populations induce similar productions of IL-2. No stimulation was observed with the CD11c$^-$ population, indicating that, as for adult, this population does not contribute to Ag presentation. The efficacy of neonate DC in presenting Ag was confirmed by the comparison of the numbers of DC necessary to stimulate hybridoma, because this experiment demonstrated that neonate DC were slightly more efficient than adult DC (Fig. 4*B*). Furthermore, the ability of neonatal DC to process Ag was assessed using a recombinant detoxified adenylate cyclase from *Bordetella pertussis* bearing the same L^d-restricted LCMV epitope (CyaA-E5-LCMV). We have previously shown that the presentation of a CD8$^+$ T cell epitope inserted into CyaA-E5 by APC is strictly dependent on the cytosolic MHC class I pathway of processing (14). Fig. 4*C* shows that the LCMV epitope is efficiently processed by neonatal DC and presented to the cell surface in association with MHC class I molecules, because these cells preincubated with CyaA-E5-LCMV stimulated specifically IL-2 production by the hybridoma MV1H7. These results indicate that neonatal DC are able to efficiently process this Ag, although in that case they appear 3–5 times less efficient than adult DC. As observed when the synthetic peptide $p_{118-126}$ is used as Ag, the CD11c$^-$ population was not able to present the LCMV epitope after incubation with CyaA-E5-LCMV. Finally, the stimulatory capacity of the H-2^d neonatal DC was assessed by assaying the numbers of DC capable of stimulating allogeneic H-2^b CD8$^+$ T cells. As shown in Fig. 5, neonatal DC appear more efficient in induction of proliferation of allogeneic CD8$^+$ T cells than do adult DC. Indeed, a higher proliferation of T cells was observed using similar numbers of neonatal DC as compared with adult DC. No proliferation was observed with syngeneic CD8$^+$ T cells, indicating the specificity of allogeneic response. Altogether, these results demonstrate the ability of neonatal DC to efficiently process and present Ag and stimulate T cells.

In vivo induction of specific CTL responses by neonatal DC in adult mice

Finally, the ability of neonatal DC to activate naive T cells and to induce in vivo-specific CTL responses was determined. Because it was previously shown that DC reach the spleen after i.v. injection (15), induction of CTL responses by neonatal DC was assessed in the spleen after i.v. immunization with peptide-pulsed DC. Freshly purified CD11c$^+$ splenocytes from 7-day-old and 8-wk-old BALB/c mice were pulsed with $p_{118-126}$. Then, 2–3 $\times$ 10^5 DC were injected i.v. to adult syngeneic mice. Seven days after the injection, splenocytes from immunized mice were stimulated in vitro with $p_{118-126}$. Five days later, CTL activity was assessed by ^{51}Cr release assay using H-2^d P815 target cells pulsed or not with the same peptide. As shown in Fig. 6, neonatal DC loaded with $p_{118-126}$ and injected into adult mice induced peptide-specific CTL responses comparable to the ones induced by adult DC, although a heterogeneity in CTL activity was observed between the different

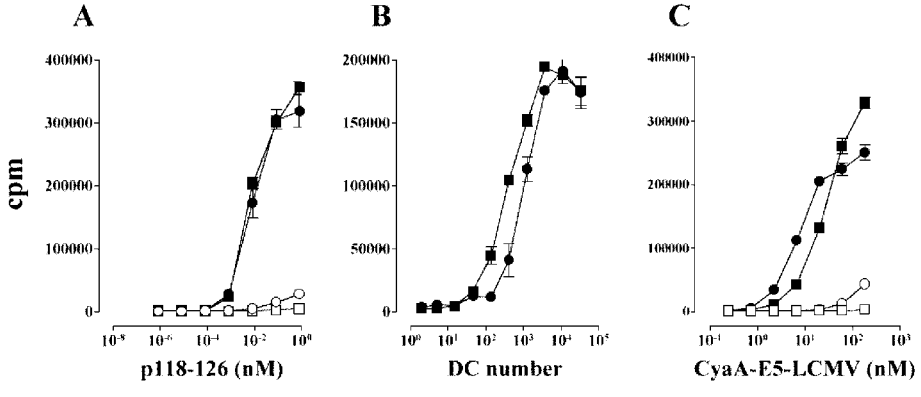

FIGURE 4. Efficient presentation of the LCMV peptide $p_{118-126}$ and CyaA-E5-LCMV to LCMV-specific CD8$^+$ T cell hybridoma by neonatal and adult DC. CD11c$^+$ cells were purified from spleen of 7-day-old (■) or 8-wk-old (●) BALB/c mice. Presentation assay was performed using 2 $\times$ 10^4 CD11c$^+$ cells incubated with various concentrations of $p_{118-126}$ (*A*) or various numbers of CD11c$^+$ cells incubated with 50 nM $p_{118-126}$ (*B*) or 2 $\times$ 10^4 CD11c$^+$ cells incubated with various concentrations of CyaA-E5-LCMV (*C*). The CD11c$^-$ splenocyte fraction of 7-day-old (□) and 8-wk-old (○) BALB/c mice was used as negative control. These cells were used as APC to stimulate the MV1H7 T cell hybridoma specific for the 118–126 LCMV epitope. IL-2 secretion by the stimulated hybridoma was determined by the CTLL-2 proliferation assay. Data represent means ± SD of duplicates and are expressed in cpm of incorporated [^{3}H]thymidine. Results are representative of three experiments.

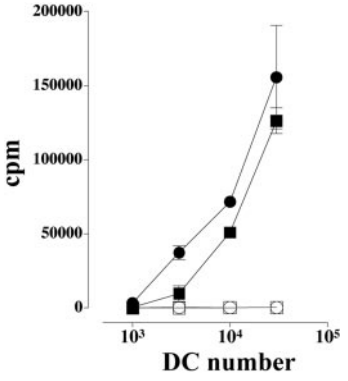

FIGURE 5. Effective in vitro priming of allogeneic T cells by neonatal DC. A fixed number of purified H-2^b $CD8^+$ T cells obtained from C56BL/6 mouse spleens was cocultured during 3 days with various numbers of H-2^d $CD11c^+$ cells isolated from 7-day-old (●) or 8-wk-old (■) BALB/c mice. As controls, purified H-2^d $CD8^+$ T cells obtained from BALB/c mice were cocultured with H-2^d $CD11c^+$ cells from 7-day-old (○) or 8-wk-old (□) BALB/c mice. Then, a proliferation assay was performed. Data represent means ± SD of duplicates and are expressed in cpm of incorporated [^{3}H]thymidine. Results are representative of three experiments.

mice tested. The low CTL responses observed after injection of neonatal and adult mice unloaded DC demonstrated the specificity of the CTL responses induced by peptide-pulsed DC. These results clearly demonstrate that in vitro pulsed neonatal DC are able to induce in vivo-specific CTL responses and thus are able to prime naive $CD8^+$ T cells in vivo.

Discussion

Although newborns are at risk of exposure to many infectious diseases, the immaturity of the neonatal immune system leads to postdone most vaccinations to 2–3 mo of age. The mechanisms responsible for such limited neonatal immune responses are not fully elucidated. The neonatal murine T cell compartment seems

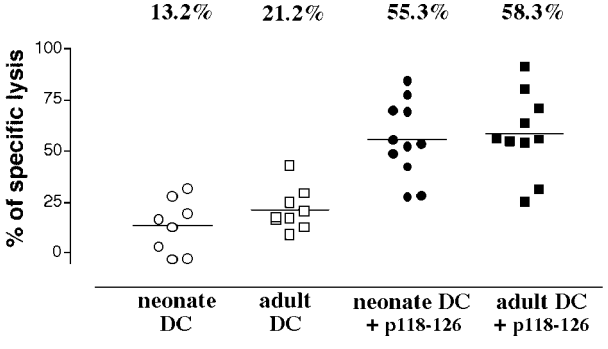

FIGURE 6. Neonatal DC induce in vivo strong specific CTL response. $CD11c^+$ cells were isolated from 7-day-old (●) or 8-wk-old (■) BALB/c mice and were pulsed with $p_{118-126}$ and transferred i.v. into 8- to 10-wk-old syngeneic mice. As controls, some mice were injected with unpulsed DC from 7-day-old (○) and 8- to 10-wk-old (□) BALB/c mice. Seven days later, mice were sacrificed, and splenocytes were restimulated in vitro with $p_{118-126}$ and irradiated syngeneic splenocytes. CTL activities were tested on ^{51}Cr-labeled P815 target cells pulsed or unpulsed with $p_{118-126}$. Results show the percentage of specific lysis obtained with P815 pulsed with the peptide corrected for background lysis obtained with unpulsed P815 target cells at an E:T ratio of 90. Background lysis was below 15% under all conditions. Each point corresponds to an individual mouse; mean values of percentage of lysis are indicated for each group of mice. Results show cumulative data from five separate experiments. Results obtained between neonatal and adult DC are not statistically different using a *t* test ($p = 0.72$).

functional because CTL and Th1 responses can be induced in neonates after immunization using strong Th-1 driving adjuvants or delivery systems (16–20). Some evidences suggest that the lack of appropriate immune T cell responses of neonates is due to the nature and quality of Ag presentation (1). The aim of this study was thus to determine the potential role of these cells in impaired neonatal immunity. For this, we analyzed the properties of DC purified from spleens of 7-day-old mice, the age which best corresponds to the main known characteristics of human neonatal immune maturation (1). Neonatal DC were purified according to the expression of the CD11c molecule which is expressed on both immature and mature DC in the spleen from adult mice. We found that this population is 12 to 50 fold less represented in neonatal spleen as compared with adult spleen. In contrast to what has been observed for neonatal macrophages (6), neonatal DC express similar levels of MHC class II and costimulatory molecules as adult DC. Moreover, our study clearly showed that neonatal $CD11c^+$ DC are functional and are able to induce in vivo $CD8^+$ T cell responses. Indeed, these cells were capable of full maturation after LPS activation. Evaluation of their Ag-presenting ability showed that these APC can process and present Ag to a $CD8^+$ T cell hybridoma and induce proliferation of allogeneic $CD8^+$ T cells in MLR assay as efficiently as adult DC. Finally, high specific cytotoxic activities were induced in vivo by in vitro pulsed neonatal DC injected to adult mice, showing the capacity of these cells to prime in vivo Ag-specific naive T cells. These last observations are in accordance with a recent paper showing that APC from 3- and 7-day-old mice induce a strong MLR from allogeneic $CD8^+$ T cells, comparable to the response obtained with adult stimulator cells (9) indicating the potentiality of neonatal DC to activate $CD8^+$ T cells. However in the same study, the authors showed a defect of neonatal APC to activate $CD4^+$ T cells due to a low expression of MHC class II molecules. By contrast, we demonstrate here that $CD11c^+$ neonatal DC express normal level of MHC class II molecules. This discrepancy could be mainly explained by difference of purification procedures used in both studies. Indeed, using cell density and adherence selective criteria, Muthukkumar et al. (9) isolated 1.7×10^5 cells per spleen from 7-day-old mice among which only 7% were $CD11c^+$ cells (corresponding to 0.12×10^5 cells from one neonatal spleen), whereas in our study, using CD11c microbeads, 4×10^5 $CD11c^+$ cells were purified per neonatal spleen (i.e., 33-fold more than in the Muthukkumar's study). This strongly suggests that cell density and adherence criteria mainly select nonfunctional progenitor cells rather than functional DC present in neonatal spleen. Thus, it is of particular interest to reinvestigate the $CD4^+$ T cell response induced by in vitro-pulsed neonatal DC expressing the $CD11c^+$ molecule to determine whether this population is really defective in inducing Th response. Furthermore, because a preferential Th2 polarization of early life responses is observed in vivo (4, 21), the Th polarization induced by neonatal $CD11c^+$ DC has to be determined.

Altogether, our results indicate that the weak Th1/CTL immune responses observed in neonates cannot be explained by an intrinsic defect in nature and quality of Ag presentation by APC, at least for the $CD8^+$ T cell responses. This limitation is also unlikely to be due to the reduced number of DC found in neonatal spleens, because the DC:T cell ratio was 0.1 as compared with 0.08 in adult spleens, suggesting that their representation is sufficient for APC-T cell interactions to take place. It has been suggested that the lack of CTL induction in neonate could be due to the biased Th2 response observed in neonates (1), and the contribution of neonatal DC to this preferential polarization remains to be determined. We previously showed that both Th and CTL responses are induced in

adult mice immunized with DC loaded with $p_{118-126}$ which contains both H-2^d class I and class II epitopes (22). The comparable LCMV-specific CTL responses induced by either neonatal or adult DC do suggest that the Th response induced by $p_{118-126}$-pulsed neonatal DC does not negatively affect the CTL response. Thus, this demonstration of the full potential capacity of neonatal murine DC to induce strong CTL responses in vivo makes them an attractive target for vaccination strategies to induce protective immunity in early life.

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