CD4-Independent Protective Cytotoxic T Cells Induced in Early Life by a Non-Replicative Delivery System Based on Virus-like Particles

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The relative immaturity of the neonatal immune system limits CD4^{+} Th1 and cytotoxic T lymphocyte (CTL) responses, and represents a significant challenge for the development of vaccines against intracellular pathogens. In this report, we demonstrate the ability of a non-replicative delivery system based on parvovirus-like particles (VLP) to induce CTL responses in the neonatal period. A single immunization of 1-week-old BALB/c mice with recombinant VLP carrying a CD8^{+} T cell determinant from lymphocytic choriomeningitis virus (VLP-LCMV) induced antigen-specific CD8^{+} cytotoxic T cells that were similar to those elicited by adult immunization, as assessed by cytotoxic activity, interferon (IFN)-γ secretion, cytotoxic precursor cell frequencies, *in vitro* avidity for antigen and protective activity against viral challenge. These CTL responses are elicited within 2 weeks of a single immunization, in the absence of adjuvant and independently of the presence and help of CD4^{+} T cells, highlighting the potential of VLP as candidate vaccine vectors in early life.   © 2003 Elsevier Science (USA)

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INTRODUCTION

The immaturity of the immune system poses severe challenges for the induction of protective immune responses in early life. Antibody responses elicited by neonatal and infant immunization are quantitatively limited, both in humans and in mice, and may be further inhibited by antibodies of maternal origin (Siegrist, 2001). Early-life T cell responses, although more readily induced than previously considered, and less susceptible to inhibition by maternal antibodies, also differ from adult responses (Adkins, 1999; Garcia *et al.,* 2001; Siegrist, 2001). In early-life murine models, limited CD4^{+} Th1 and CD8^{+} cytotoxic T cell (CTL) responses were observed following infection (Sarzotti *et al.,* 1996) or after immunization with conventional measles or hepatitis B vaccines (Barrios *et al.,* 1996b; Brazolot Millan *et al.,* 1998). In human infants, disease severity of neonatal herpesvirus infection is associated with limited interferon (IFN)-γ responses (Burchett *et al.,* 1992; Hayward *et al.,* 1984), and the higher relative risk of chronicity following acute hepatitis B infection in infants and young children reflects their failure to clear virus (Hyams, 1995). Data on

infant T cell responses to immunization is still scarce. Although BCG immunization induces adult-like neonatal IFN-γ responses (Vekemans *et al.,* 2001), IL-12 and IFN-γ responses to live attenuated measles vaccine remain limited up to one year of age, as compared to adults (Gans *et al.,* 2001; Gans *et al.,* 1999). Altogether, these observations identified limitations of CD4^{+} Th1 and CTL responses as characteristic features of neonatal immunity. This has been suggested to reflect a preferential differentiation of early-life T cell responses toward the Th2 pathway, as a "default" developmental pathway (Fadel and Sarzotti, 2000; Adkins, 2000).

These limitations represent a significant challenge for the development of vaccines against important intracellular pathogens, including tuberculosis, malaria and HIV, such that there is a requirement for novel antigen delivery systems able to induce adult-like CD4^{+} Th1 and CTL responses in early life. In the last few years, DNA vaccines (Bot, 2000), live replicating vaccines (Franchini *et al.,* 2001; Kovarik *et al.,* 2001; Siegrist, 2001), and a few strong Th1-driving adjuvants (Barrios *et al.,* 1996a; Brazolot Millan *et al.,* 1998; Forsthuber *et al.,* 1996; Kovarik *et al.,* 1999) were found to ameliorate these limitations. It was thus suggested that the induction of adult-like CD4^{+} Th1 and CTL neonatal responses essentially depends on the extent of neonatal APC activation and/or of neonatal APC-T cell interactions (Adkins, 1999). Whether the main challenge is that of a higher activation threshold of neonatal APC, of suboptimal CD4^{+} T cell neonatal re-

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sponses (Brugnoni et al., 1994; Durandy et al., 1995; Fuleihan et al., 1994; Nonoyama et al., 1995) and/or of other determinants of neonatal APC-T cell interactions is currently unknown.

A novel non-replicative virus-like particle (VLP) delivery system, based on the self-assembly of the modified porcine parvovirus (PPV-VLP) VP2 capsid protein, was recently reported as inducing strong MHC class I-restricted CTL responses in adult mice, in the absence of adjuvant, independently of CD4^{+} T cell help (Sedlik et al., 1997), and thus likely resulting from direct APC maturation/activation (Bennett et al., 1998; Moron et al., 2002; Schoenberger et al., 1998). We used this delivery system to test whether non-replicative antigens can induce neonatal CTL responses, and to evaluate the requirement of CD4^{+} T cell help in the induction of neonatal CTL responses. Experiments were performed with PPV-VLP into which the LCMV$_{118-132}$ peptide, which contains both a weak MHC class II-restricted and a strong immunodominant MHC class I-restricted determinant from the LCMV nucleoprotein of the WE strain (Aichele et al., 1990; Fayolle et al., 1996), was inserted (VLP-LCMV). Comparative analyses of humans and mice have indicated that the stage of immune maturation that prevails during the neonatal period (defined as 28 days in humans) is best approximated by that of 1-week-old mice (Siegrist, 2001). Induction of anti-viral CD8^{+} T cell responses and protective efficacy against viral challenge were therefore compared following VLP-LCMV immunization of 1-week-old and adult mice, in presence or absence of CD4^{+} T cells.

RESULTS

Induction of adult-like bulk cytotoxic responses by neonatal VLP-LCMV immunization

BALB/c mice were immunized i.p. at the age of 1 week and as adult controls with a single dose (10 μg) of VLP-LCMV or control VLP (VLP-ctrl). Neonatal immunization was well tolerated and did not affect weight gain, a sensitive marker of vaccine-induced toxicity in early life. Two weeks after priming, splenocytes were restimulated for 5 days in vitro with the LCMV$_{118-132}$ peptide and assessed for their capacity to lyse LCMV$_{118-132}$-pulsed P815 target cells. A strong CTL response was observed both in mice immunized at the age of 1 week and in adult controls, whereas no lysis was observed with splenocytes from mice immunized with VLP-ctrl (Fig. 1), or on target cells pulsed with a control peptide, a CTL determinant from measles virus hemagglutinin (MVHA$_{544-552}$, not shown). As P815 target cells do not express MHC class II nor are susceptible to NK cell lysis, CD4^{+} T cells and NK cells are unlikely to contribute significantly to the observed cytotoxicity. Since CTL responses against LCMV$_{118-132}$ even in adults require repeated injection of the peptide with adjuvant (iFA or cFA) (Fayolle et al., 1991), the observed CTL responses after single immuni-
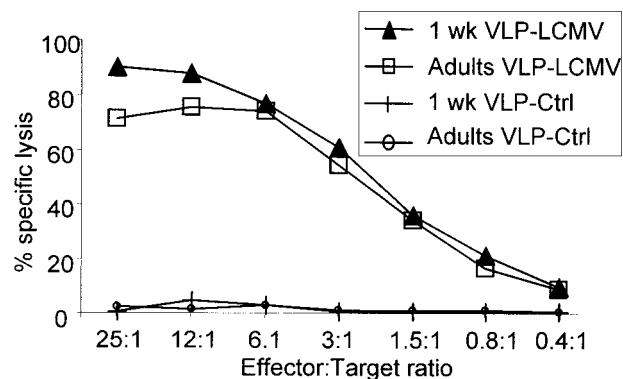

FIG. 1. CD8^{+} T cell cytotoxic responses to neonatal and adult VLP-LCMV immunization. One-week-old and adult BALB/c mice were immunized i.p. with 10 μg of VLP-LCMV or VLP-ctrl. Two weeks after immunization, splenocytes were harvested, pooled by immunization groups and restimulated in vitro for 5 days with the LCMV$_{118-132}$ peptide. Cytotoxic activity was measured with ^{51}Cr-labeled P815 targets cells pulsed with 50 μM of LCMV$_{118-132}$ peptide (P815- LCMV$_{118-132}$), or incubated with medium alone (P815-medium). Data represent the means of percent specific lysis ((% lysis P815-LCMV$_{118-132}$) − (% lysis P815-medium)) from duplicate wells obtained at each effector/target ratio. Results are from 1 of 3 representative experiments. Lysis of uncoated target cells by all effectors was less than 10% at all E:T ratios and is not shown.

zation without adjuvant are likely to be due to the PPV-VLP antigen delivery system.

Similar frequencies and avidity of antigen-specific effector cells induced in 1-week-old and adult mice

CTL responses were characterized and quantified by limiting dilution analysis (LDA) and assessment of the frequency of IFN-γ-producing cells by ELISPOT. LDA performed 2 weeks after immunization indicated that the frequency of precursors developing into cytotoxic effectors (CTLp) was high (>1/10,000) both in mice immunized with VLP-LCMV at 1 week of age and as adults (Table 1), whereas frequencies remained lower than 1/50,000 following VLP-ctrl immunization. Although the frequency of CTLp varied between LDA experiments, a threefold lower CTLp frequency (expressed per total spleen cells) was consistently observed following neonatal immunization ($P < 0.05$). Quantification of the total number of CTLp per spleen confirmed that, 2 weeks after priming, the number of LCMV-specific CTLp was threefold lower following neonatal compared to adult immunization (Table 1). However, flow cytometric analysis indicated that CD8^{+} T cells present in the spleen of 3-week-old BALB/c mice (i.e., at the time of evaluation of CD8^{+} T cell responses) represent 5–7% of total splenocytes, compared to 11–13% in adult mice (data not shown). Thus, the frequency within the CD8^{+} T cell subset of splenocytes able to generate cytotoxic effectors against LCMV$_{118-132}$ was not significantly different following early-life and adult VLP-LCMV immunization (Table 1).

TABLE 1

CTLp Frequencies after VLP-LCMV Immunization[a]

	CTLp frequency[b]	Total CTLp/spleen[c]	CTLp/CD8+ T cells[c]
Exp 1: VLP-LCMV			
1 week	1/5227	12627	1/261
Adults	1/1597	55103	1/192
Exp 2: VLP-LCMV			
1 week	1/4509	14194	1/225
Adults	1/1733	46163	1/208
Exp 3: VLP-LCMV			
1 week	1/9263	6261	1/463
Adults	1/3623	20425	1/435
Exp 4: VLP-LCMV ± CD4-depletion[d]			
1 week Control	1/8924	7396	1/446
1 week CD4-depleted	1/11242	5960	1/562

[a] One-week-old and adult mice were immunized with 10 μg of VLP-LCMV. CTLp frequencies were assessed 2 weeks later, after *in vitro* restimulation, as described in Material and Methods.

[b] CTLp frequencies were expressed as the mean of the frequencies obtained in various immunization groups.

[c] Total CTLp per spleen and the frequency of LCMV-specific CTLp per CD8+ T cells were calculated based on total numbers of splenocytes or CD8+ T cells present at time of sacrifice, respectively.

[d] For *in vivo* depletion of CD4+ T cells (Exp. 4), mice were injected i.p. with 300 μg of anti-CD4 (GK1.5) mAb on days −1, 0, +1, +7 and +11 of the immunization scheme, and results were similar in a second experiment.

Similar results were obtained by IFN-γ ELISPOT analysis. Under our experimental conditions, *ex vivo* ELISPOT analyses did not detect any LCMV$_{118-132}$-specific IFN-γ spot forming cell (SFC) 2 weeks after a single immunization of adult or 1-week-old mice. This is in contrast to a previous report where immunization included the administration of 2 vaccine doses to adult mice (Sedlik *et al.,* 1997). However, similarly high frequencies of LCMV$_{118-132}$-specific IFN-γ SFC in both age groups were observed after bulk *in vitro* restimulation of splenocytes with the LCMV$_{118-132}$ peptide (Fig. 2). Since LCMV$_{118-132}$ comprises both an MHC class II- as well as an MHC class I-restricted determinant, it was important to define the cell subset responsible for this IFN-γ-secretion. Depletion of CD8+ T cells during *in vitro* restimulation abrogated IFN-γ responses, confirming CD8+ T cells as the source of IFN-γ-SFC in neonatally immunized mice (Fig. 2), as previously observed in adults (Sedlik *et al.,* 2000). Immunization with VLP-ctrl did not elicit IFN-γ-producing LCMV$_{118-132}$-specific T cells (Fig. 2). IL-5-producing LCMV-specific SFC were not observed in mice from any group (not shown).

Since the *in vivo* efficiency of CTL does not only depend on CTL numbers but also on their avidity for their antigen-bearing target (Alexander-Miller *et al.,* 1996; Sedlik *et al.,* 2000), we next compared the capacity of adult and neonatal effector cells to lyse P815 targets cells
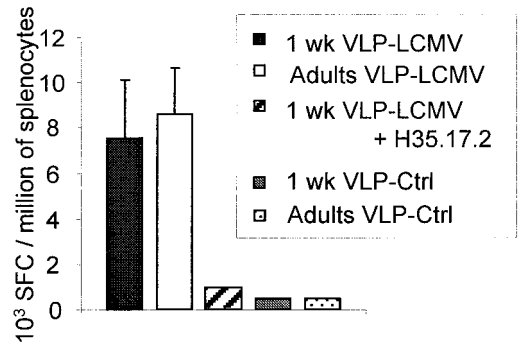

FIG. 2. Frequency of IFN-γ-secreting CD8+T cells following neonatal and adult VLP-LCMV immunization. One-week-old and adult BALB/c mice were immunized i.p. with 10 μg of VLP-LCMV or VLP-ctrl. Two weeks after immunization, splenocytes were harvested and restimulated *in vitro* during 4 days with LCMV$_{118-132}$, prior to analysis of the frequency of IFN-γ-secreting cells in individual mice by ELISPOT, as described in Material and Methods. When indicated, 10 μg/ml of anti-CD8 (H35.17.2) mAb was added into the ELISPOT plates. Data are expressed as the number of SFC per million of splenocytes in wells containing LCMV$_{118-132}$ with SFC in wells without LCMV$_{118-132}$ subtracted. Error bars depict standard deviations from 6 to 8 mice. Results are representative of 3 experiments. SFC in wells without LCMV$_{118-132}$ were less than 5.

pulsed with a range of LCMV$_{118-132}$ concentrations. As shown in Fig. 3, immunization with VLP-LCMV primed early-life CTL whose restimulated effector cells were of similar avidity for antigen-pulsed targets as in adults. Previous experiments comparing *in vitro* CTL avidity of adult mice immunized with various antigen delivery systems have indicated that this reactivity profile reflects a strong avidity for target cells and correlates with *in vivo* protection (Sedlik *et al.,* 2000).
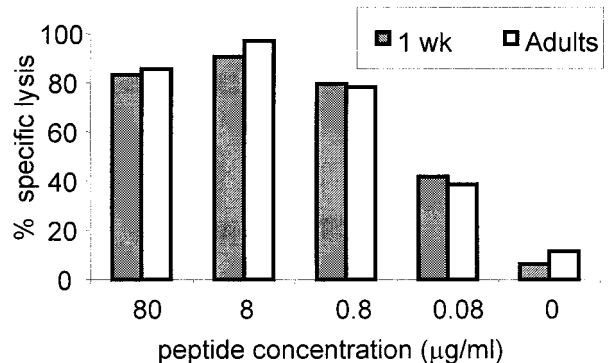

FIG. 3. Avidity of adult and early-life LCMV-specific CTL. Two weeks after VLP-LCMV immunization of one-week-old or adult mice, splenocytes were pooled by immunization group and restimulated *in vitro* with the LCMV$_{118-132}$ peptide as in Fig. 1. Cytotoxic activity was measured with ^{51}Cr-labeled P815 targets cells pulsed with various concentrations of LCMV$_{118-132}$ peptide. Data represent the means of percent specific lysis from duplicate wells obtained at a 6:1 effector/target ratio. Results are representative of two experiments.

CTL responses to neonatal VLP-LCMV immunization are CD4$^+$ T cell-independent

CD4$^+$ T cells, and in particular via CD40–CD40L interaction, play an important role in inducing CTL responses by activating APC through costimulation (Bennett et al., 1998; Schoenberger et al., 1998). To assess the role of neonatal CD4$^+$ T cells in the activation of neonatal CTL responses, we asked whether early-life CTL responses to VLP-LCMV could be induced in the absence of CD4$^+$ T cells in vivo. One-week-old mice were injected i.p. with 300 μg of anti-CD4$^+$ (GK1.5) MAb on days -1, 0, +1, +7 and +11 of the immunization schedule (Buller et al., 1987). This resulted in >90% depletion of CD4$^+$ T cells in GK1.5-treated mice (not shown). CTL responses were analyzed under limiting dilution conditions on Day 14 after immunization (as described in Material and Methods). In vivo CD4$^+$ T cell-depletion did not abrogate LCMV-specific CTL responses, and CTLp remained statistically similar to those induced in control non-depleted 1-week-old mice (Table 1, exp. 4). Therefore, as previously demonstrated in adults (Sedlik et al., 1997), early-life immunization with VLP-LCMV induces CD4$^+$ T cell-independent CTL responses even at 1 week of age.

Antiviral protection through neonatal VLP-LCMV immunization

Are neonatally induced CTL efficient in protecting immunized mice from viral challenge? This question was assessed by challenge with LCMV-WE two weeks after immunization of 1-week-old and adult BALB/c mice. Mice were challenged intravenously with 200 pfu of LCMV-WE strain and sacrificed 4 days later for determination of splenic virus titers (Wirth et al., 2000). As expected, high virus titers were observed in all mice immunized with VLP-ctrl, regardless of age at immunization (Fig. 4). In contrast, viral titers were strongly reduced in mice immunized with VLP-LCMV at 1 week of age or as adults (Fig. 4). The majority of immunized mice were free of detectable virus in the spleen. Thus, neonatally induced anti-LCMV$_{118–132}$ T cells have the same protective potential against viral challenge as those induced in adult life.

DISCUSSION

In this study, we demonstrate that neonatal immunization with a non-replicating parvovirus-like particle delivery system induces antigen-specific CD8$^+$ cytotoxic T cells that are similar to those elicited by adult immunization in all functional aspects amenable to analysis: cytotoxic activity, frequency of IFN-γ-secreting cells, cytotoxic precursor cell frequencies, in vitro avidity for antigen and protection against viral challenge. These CTL responses are elicited within 2 weeks of a single immunization, in the absence of adjuvant and in the absence of CD4$^+$ T cells.
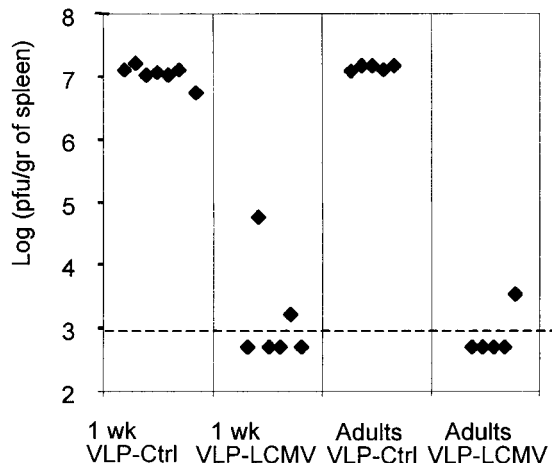

FIG. 4. Protective efficacy of VLP-LCMV immunization against LCMV-WE challenge. One-week-old and adult mice were immunized i.p. with 10 μg of VLP-LCMV or VLP-ctrl. Two weeks after immunization, mice were injected intravenously with 200 pfu of LCMV-WE. LCMV-WE viral titers were assessed in the spleen 4 days after challenge, as described in Material and Methods. Virus titers are expressed as plaque forming units (pfu)/gram of spleen tissue. Each diamond represents an individual mouse. The dotted line represents the assay cutoff. Results are representative of 2 experiments.

In recent years, DNA vaccines (Bot, 2000) and live replicating viral vaccines (Franchini et al., 2001; Kovarik et al., 2001; Siegrist, 2001) were shown to circumvent the limitations of early-life CTL responses in murine models of neonatal and early life immunization, in contrast to inactivated or non-replicating viral vectors. This was also achieved by certain adjuvants, such as CpG-oligonucleotides (Brazolot Millan et al., 1998; Kovarik et al., 1999) which are capable of strong neonatal APC activation via Toll-like-receptor-mediated recognition (Hemmi et al., 2000). We report here, for the first time, that adult-like antiviral CTL responses may be elicited in early life by a non-replicating antigen-delivery system, without the need for adjuvant. VLP-LCMV immunization induced strong cytotoxic responses, characterized by similar frequencies of LCMV$_{118–132}$-specific CTL precursors, as well as similar frequencies of IFN-γ-producing CD8$^+$ T cells following neonatal and adult immunization. This may be an important point for adult-like viral clearance since small differences in antigen-specific CD8$^+$ T cell numbers have been demonstrated to lead to an inability to clear polyoma virus infection in susceptible newborn mice (Moser et al., 2001). Neonatally induced CTL were also qualitatively similar to those induced in adults, as demonstrated by their IFN-γ-production and their similar in vitro avidity for peptide-pulsed target cells, an important determinant of protective capacity (Alexander-Miller et al., 1996; Derby et al., 2001; Sedlik et al., 2000; Speiser et al., 1992). Importantly, they were already detected, and protective, 14 days after a single immunization. In contrast to previous reports using DNA vaccines or replicating vectors which persist in vivo during several weeks,

and are thus still present at time of greater immune competence, or to reports only assessing neonatal induction of CTL responses at several weeks of age, our observations demonstrate unequivocally the competence of VLP-induced neonatal T cells to rapidly mount protective responses, an essential element for the induction of protection as soon as possible after birth.

A potential limitation of our study is that we were unable to detect LCMV-specific CD8$^+$ T cells directly *ex vivo*, whether by assessing cytotoxicity, intracellular IFN-γ producing cells or tetramer-binding cells (not shown), either in adults or neonates. This is in accordance with previous observations of the much lower frequency of CD8$^+$ T cells elicited by a single immunization with non-replicating vaccines compared to viral infection. We thus cannot exclude that *in vitro* restimulation did not modulate CD8$^+$ T cell frequency and/or characteristics. However, regardless of the assay used, CTL responses assessed in neonates and in adults were always similar. In addition, the true functional competence of neonatal CD8$^+$ T cells is directly demonstrated by their *in vivo* protective capacity.

Why are PPV-VLP so successful in the generation of early-life CTL responses? VLP are promising novel vaccine candidates, combining high immunogenicity with lack of detectable toxicity in the absence of adjuvants, while inducing neutralizing antibody and CTL responses in a number of murine model systems (Breitburd *et al.,* 1995; Kirnbauer *et al.,* 1992; Sedlik *et al.,* 2000; Sedlik *et al.,* 1999; Sedlik *et al.,* 1997; Suzich *et al.,* 1995). Early clinical trials have already confirmed some of these properties in humans (Ball *et al.,* 1999; Evans *et al.,* 2001; Harro *et al.,* 2001). One important feature of PPV-VLP is their ability to ablate the requirement for adjuvantation as well as for CD4$^+$ T cell help in adult mice (Sedlik *et al.,* 2000). We show here that this is also true in the immature immune system. LCMV$_{118-132}$-specific CD4$^+$ T cell cytokine responses were not detected in our assays (data not shown), despite the fact that the LCMV$_{118-132}$ peptide contains a weak CD4$^+$ T cell determinant as well as its strong CD8$^+$ T cell determinant. In any case, VLP-specific or LCMV-specific CD4$^+$ T cells, if present, are not required for the induction of LCMV$_{118-132}$-reactive CTL, as demonstrated by the lack of inhibition by *in vivo* CD4$^+$ T cell-depletion in early-life (our data) or adult (Sedlik *et al.,* 2000) CTL responses.

The remarkable capability of PPV-VLP to ablate the requirement for CD4$^+$ T cell help in adult mice has been hypothesized to be due to a strong and direct activation of APC, in particular DC (Lo-Man *et al.,* 1998; Sedlik *et al.,* 2000). Human papillomavirus-like particles were recently shown to be efficiently internalized by adult DC, inducing their phenotypic maturation and production of inflammatory cytokines (Lenz *et al.,* 2001). We have found this to be also true for the PPV-VLP used in our study. Indeed, *in vivo*, PPV-VLPs are very efficiently captured by dendritic cells and induce phenotypic maturation as well as expression of new cell surface markers (Moron *et al.,* 2002).

Induction of adult-like Th1 and CTL neonatal responses has been suggested to depend on the extent of neonatal APC activation and/or of neonatal APC-T cell interactions (Adkins, 1999). LPS-activation of cord-blood derived human DC results in limited IL-12 production (Goriely *et al.,* 2001) and could lead to preferential induction of CD4$^+$ Th2 neonatal responses and/or limitation of Th1 responses, therefore inhibiting CTL induction. On the other hand, it has recently been demonstrated that purified neonatal DCs are perfectly able, when loaded with a synthetic peptide containing a CTL epitope and injected to adult mice, to efficiently prime specific CTL responses (Dadaglio *et al.,* 2002). Our results suggest that neonatal APC may be stimulated *in vivo* by PPV-VLP so as to induce adult-like CTL responses, even without CD4$^+$ T cell help. Another hypothesis is that limitations of early-life CTL responses to conventional vaccines may result from an active inhibition/deviation mediated by the neonatal environment, such as through preferential induction of CD4$^+$ Th2 neonatal cytokine responses. Our preliminary observations with another—CD4$^+$ T cell dependent—antigen delivery system suggest that neonatal CTL responses are indeed susceptible to inhibition by concomitantly induced CD4$^+$ Th2 responses (manuscript in preparation). Whether the limited induction of neonatal CTL responses to conventional antigens result from activation of neonatal APC and/or from limited or deviated CD4$^+$ T cell responses, the APC activation capacity and CD4$^+$ T cell independence of PPV-VLP identifies them as safe and potent antigen delivery systems for induction of neonatal and early-life cytotoxic responses to intracellular pathogens.

MATERIALS AND METHODS

Mice, virus, and peptide

Specific pathogen-free adult BALB/c inbred mice were purchased from Biological Research Laboratories (Fülinsdorf, Switzerland), kept under specific pathogen free conditions at the W.H.O. Center for Neonatal Vaccinology, and manipulated according to National and European guidelines. Breeding cages were checked daily for new births, and the day of birth was recorded as the day the litter was found. Pups were kept with mothers until weaning at the age of 4 weeks. Adult mice were used at 8–12 weeks of age. LCMV strain WE was originally obtained as triple-plaque-purified stock from Dr. F. Lehmann-Grube (Heinrich-Pette Institut, Hamburg, Germany). The RPQASGVYMGNLTAQ synthetic peptide carrying the p118–132 sequence from the LCMV strain WE nucleoprotein (Adkins, 1999; Schulz *et al.,* 1989) was obtained commercially.

Preparation of chimeric VLP expressing the LCMV p118–132 peptide

The recombinant VLP-LCMV and control VLP were obtained from Ingenasa (Madrid, Spain) after construction, production and purification as previously described in details (Sedlik *et al.*, 1997). Briefly, the PPV:VP2 gene was expressed either with the LCMV p118–132 peptide sequence (VLP-LCMV) or without this sequence (VLP-ctrl) in a baculovirus vector system. After infection of Sf9 insect cells, VLPs were purified by salt precipitation with 20% ammonium sulfate followed by dialysis. Characterization of VLP-LCMV and VLP-ctrl obtained by CsCl sedimentation analysis and electron microscopy revealed properties identical to those of native virions. The molecular weight of the LCMV peptide represents 3% of that of VLP-LCMV.

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

Mice were immunized i.p. in groups of 6 to 8 at 1 week of age (neonatal immunization) or as adults (controls), with a single injection of 10 μg of VLP-LCMV or VLP-ctrl in the absence of adjuvant. Where indicated, mice were injected i.p. with 300 μg of anti-CD4 (GK1.5) mAb prepared from ascitic fluid (Swain *et al.*, 1984) 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

Splenocytes were harvested 2 weeks after immunization. Equal numbers of splenocytes from individual mice were pooled and cultured as bulk or under limiting dilution conditions as described (Barrios *et al.*, 1996b). Briefly, control bulk cultures containing 50×10^6 splenocytes from immune mice were incubated with 25×10^6 irradiated (2500 rad) syngenic spleen cells and 2 μM of LCMV$_{118-132}$ in RPMI 1640, supplemented with HEPES, amino acids, antibiotics, and 10% fetal calf serum (medium). They were tested on Day 5 by adding varying number of effector cells to 10^5 ^{51}Cr-labeled P815-pulsed (H-2^d) target cells pulsed with 50 μM of LCMV$_{118-132}$ or incubated in medium alone. After 5 h of incubation, cell culture supernatants were harvested for determination of activity in a γ-counter. The percentage of specific lysis was calculated as ((experimental c.p.m. $-$ spontaneous c.p.m.)/(total c.p.m.$-$ spontaneous c.p.m.)) $\times$100. Data shown are averages from duplicate wells. Standard deviation from the average was always <5%. Spontaneous release and total release were determined from target cells incubated with medium alone or after the addition of 100 μl of 1M HCl, respectively. For limiting dilution assay, varying numbers of responding cells were dispensed into 96 round-bottomed microwells together with 5 $\times 10^5$ irradiated syngeneic stimulator spleen cells, 2 μM/ml of LCMV$_{118-132}$ CTL peptide, and medium with EL-4 supernatant as a source of IL-2 (final IL-2 concentration: 30 Units/ml). Fresh medium containing IL-2 was added on Day 7. Individual wells were tested on Day 10 for CTL activity. ^{51}Cr-labeled (H-2^d) P815 target cells (10^5) were added to each well for a 5-h incubation at 37°C. Spontaneous release and total release were determined from target cells incubated with medium alone or after the addition of 100 μl of 1M HCl, respectively. Cell supernatants were harvested for determination of radioactivity in a gamma counter. Wells with a ^{51}Cr-release content superior to the mean value $+5$ SD of the radioactivity measured in the supernatant of targets cells alone were counted as positive wells. CTL precursor (CTLp) frequencies, which represent the mean frequency for the mice whose cells were initially pooled, were determined by the intersection of the regression line of the frequency of negative wells with the cut-off frequency of 37% (Barrios *et al.*, 1996b). The CTLp total numbers and the frequency of LCMV-specific CD8$^+$ T cells were calculated based on the numbers of splenocytes and of CD8$^+$ T cells (identified by FACS analyses), respectively, from spleens of mice at time of challenge (3 weeks, adults).

ELISPOT assay of LCMV$_{118-132}$-specific CD8$^+$ T cells producing IFN-γ

The ELISPOT assay was adapted from Miyahira *et al.* (Miyahira *et al.*, 1995) for detection of LCMV-specific IFN-γ secreting T cells. Nitrocellulose microplates (Millipore) were coated with 5 μg/ml rat anti-mouse IFN-γ antibody (clone R46A2, Pharmingen). Spleen cells were restimulated *in vitro* with LCMV$_{118-132}$ during 4 days, washed and dispensed at four twofold dilutions in ELISPOT wells in medium containing 30 U/ml of IL-2 (EL-4 supernatant). The cells were exposed to 10^5 irradiated (8000 rad) MHC class II negative P815 cells that had been pulsed with 50 μM of LCMV$_{118-132}$ or medium alone (control P815) prior to a 24–28 h incubation at 37°. When indicated, 10 μg/ml of anti-CD8$^+$ (H35.17.2) mAbs (Pierres *et al.*, 1982) was added into the wells. The plates were then washed, incubated overnight at 4°C with 1 μg/ml biotinylated anti-mouse IFN-γ antibody (clone XMG, Pharmingen) followed by peroxydase-conjugated Extravidin. Spots were developed by adding freshly prepared substrate buffer (0.3 mg/ml of 3 amino-9-ethyl-carbazole and 0.03% H_2O_2 in 0.1 M sodium acetate pH 4.8). IFN-γ spot forming cells (SFC) were counted using the KS ELISPOT System (Zeiss, Hallbergmoos, Germany) and expressed as spots/million spleen cells.

Assessment of protective antiviral effect

BALB/c mice were injected intravenously with 200 plaque-forming units (pfu) of LCMV-WE. Four days after challenge, mice were sacrificed and virus titer deter-

mined in the spleen as described elsewhere in details (Wirth *et al.,* 2000). Briefly, spleens were homogenized in 4 ml glass tubes and spun at 1700 g for 10 min at 4°C. Serial 10-fold dilution of homogenized supernatants were incubated in 24-well plates coated with the adherent fibrosarcoma MC57G cell line. After absorption and 48 h of culture under methylcellulose overlay, cellular monolayers were fixed, permeabilized, and stained with monoclonal rat anti-LCMV antibody and peroxidase-labeled second step antibody. Virus titers are expressed as pfu per gram of spleen. The detection limit for this plaque assay was 0.5 pfu/mg spleen.

Statistical Analysis

Significance analysis between results obtained from various groups of mice was performed by using the Mann–Whitney U test. Probability values > 0.05 were considered insignificant.

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