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Immunization of neonatal mice with *LAMP*/p55 HIV *gag* DNA elicits robust immune responses that last to adulthood

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ABSTRACT

Successful T cell priming in early postnatal life that can generate effective long-lasting responses until adulthood is critical in HIV vaccination strategies because it prevents early sexual initiation and breastfeeding transmission of HIV. A chimeric DNA vaccine encoding p55 HIV *gag* associated with lysosome-associated membrane protein 1 (*LAMP-1*; which drives the antigen to the MIIC compartment), has been used to enhance cellular and humoral antigen-specific responses in adult mice and macaques. Herein, we investigated *LAMP-1*/*gag* vaccine immunogenicity in the neonatal period in mice and its ability to generate long-lasting effects. Neonatal vaccination with chimeric *LAMP*/*gag* generated stronger Gag-specific immune responses, as measured by the breadth of the Gag peptide-specific IFN-γ, proliferative responsiveness, cytokine production and antibody production, all of which revealed activation of CD4+ T cells as well as the generation of a more robust CTL response compared to *gag* vaccine alone. To induce long-lived T and B cell memory responses, it was necessary to immunize neonates with the chimeric *LAMP*/*gag* DNA vaccine. The *LAMP*/*gag* DNA vaccine strategy could be particularly useful for generating an anti-HIV immune response in the early postnatal period capable of inducing long-term immunological memory.

Introduction

Pediatric infection with HIV remains a significant public health problem in several developing countries, particularly in Sub-Saharan Africa, where high HIV-seroprevalence exists among pregnant women and more than 2 million children are living with HIV/AIDS (UNAIDS, 2008). In addition to perinatal HIV-1 transmission, changes in sexual behavior and drug use are also serious social problems that likely contribute to the high rate of HIV-1-infected children less than 15 years of age (UNAIDS, 2008). In untreated populations, approximately 20% of children infected with HIV-1 rapidly progress to AIDS or death in the first year of life (Blanche et al., 1997; Mayaux et al., 1996). Therefore, there is an emerging need for studies involving anti-HIV vaccine formulations capable of being immunogenic and eliciting a long-lasting immune response in the neonatal period and throughout childhood.

The neonate adaptive immune response shows expressive variability, ranging from high antigen non-responsiveness and denominated immunological tolerance to fully mature and functioning responses, and it usually presents susceptibility to viral infections (Siegrist, 2001; Adkins et al., 2004; Rigato et al., 2009; Sarzotti et al., 1996; Forsthuber et al., 1996). Several peculiarities contribute to the relative immaturity of the immune system in the neonatal phase, including small numbers of lymphoid cells, delayed germinal center formation on lymphoid organs and functional impairment of T, B and dendritic cells (DCs). These features result in delayed cellular/humoral responses of shorter duration and lower peak Ab levels (Adkins et al., 2004; Rigato et al., 2009; Siegrest, 2001; Adkins, 2003. In fact, neonatal DCs are not good inducers of Ag-specific T cell proliferation due to inadequate expression of costimulatory and MHC class II molecules and deficient IL-12 production (Pihlgren et al., 2001; Min et al., 2001; Muthukkumar et al., 2000; Rose et al., 2007). Furthermore, the neonatal phase is characterized by a Th2-skewed Ag-specific immune response (Simpson et al., 2003). Despite all of the limitations and impairments of the immune system, it has been shown that when vaccines are administered in ideal conditions, neonatal mice can generate vigorous adult-like CTL responses rather than become tolerant (Siegrist, 2007; Brito et al., 2009).

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DNA-based vaccination in the neonatal period is a strategy for generating immunity to viral infections in mice by intracellular pathogens (Siegrist, 2007; Zhang et al., 2002), even in the presence of inhibitory maternal antibodies (Martinez et al., 1997; Sedegah et al., 2003; Capozzo et al., 2006). Usually, antigenic peptides generated after DNA cell transfection are processed in the antigen presenting cell (APC) cytoplasm and preferentially presented through MHC class I molecules (Stevenson, 2004). DNA vaccines in humans were significantly less immunogenic according to results obtained from preclinical studies (Lu et al., 2008). The strengthening of promoters, enhancers and/or other transcriptional elements in DNA vaccines (Calarota and Weiner, 2004), including biologically active components such as chemokines and cytokines encoded in the DNA (Lu et al., 2008; Cheng et al., 2001), and improving peptide presentation by MHC class II molecules (Marques et al., 2003; Bennett et al., 1997) are all strategies that have been used to enhance the immunogenicity of genetic vaccines.

DNA vaccines encoding chimeric antigens in frame with the lysosome-associated membrane protein (LAMP) are able to target the antigen to the MHC class II-rich endosomal/lysosomal compartments (MIIC) and thereby enhance antigen-specific immune responses (Marques et al., 2003; Chikhlikar et al., 2004; Chikhlikar et al., 2006; de Arruda et al., 2004, 2006). A chimeric DNA vaccine containing the sequence of the 55 kDa HIV-1 *Gag* protein (p55Gag) inserted between the transmembrane and cytoplasmic domains of *LAMP-1* (*LAMP*/*gag*) and flanked by the inverted terminal regions of adeno-associated virus (Chikhlikar et al., 2004; Arruda et al., 2006) has been shown to induce potent CD4+ and CD8+ T cell responses, to increase Ab production and to lead to a long-lasting immune response in adult BALB/c mice, as compared to *p55gag* native DNA (Marques et al., 2003; Chikhlikar et al., 2006; Arruda et al., 2004, 2006). Furthermore, vaccination of Rhesus macaques with human *LAMP*/*gag* promoted stronger cellular and humoral anti-Gag responses (Chikhlikar et al., 2006 and Valentin et al., 2009). In addition, immunization of HLA-DR4 transgenic mice with immature dendritic cells transfected with LAMP/gag induced HIV Gag-specific T cell responses of greater avidity and a broader repertoire, providing a broadly applicable strategy for the functional activation of T cell antigens for HIV therapeutic vaccines (Simon et al., 2010).

The chimeric *LAMP*/*gag* DNA vaccine could represent a strategy for overcoming the immunological tolerogenic status of the early life phase by inducing an immune response in the neonatal period. The present study was designed to test the hypothesis that the *LAMP*/*gag* DNA chimeric vaccine can prime T and B cells in neonates better than conventional DNA vaccines. If this approach could bypass the immunological immaturity in the postnatal period, it could generate long-lasting cellular and humoral immune responses and anti-HIV immunity. Here, we show that neonatal immunization with *LAMP*/*gag* DNA is better than conventional *gag* native DNA in generating strong and T and B cell response at an early age of life and persisting until adulthood.

Results

Broad Gag-specific IFN-γ and IgG responses following neonatal vaccination with LAMP/gag

We analyzed the potential immunogenicity of a *LAMP*/*gag* DNA vaccine in the neonatal period and the duration of its effect after neonatal priming. It has been previously shown that *LAMP*/*gag* DNA immunization elicits higher anti-Gag immune responses in adult mice than does the *gag* DNA vaccine, especially when a prime–boost schedule of priming with *LAMP*/*gag* and boosting with *gag* is used (*LAMP*/*gag*+*gag*) (Marques et al., 2003; Chikhlikar et al., 2006; de Arruda et al., 2004, 2006). To assess the immunogenicity of the chimeric *LAMP*/*gag* DNA vaccine in the early stages of life, the immunization protocol consisted of priming neonate mice at seven days of age (do) with *LAMP*/*gag*, *gag* or *Lamp*, boosting 18 days later and evaluating ten days after the boost. As a positive control, we immunized adult mice twice intradermally with 50 μg of *LAMP*/*gag* and *gag* plasmids (Arruda et al., 2004). Studies have shown that the characteristics of the immune response of a neonate at 7 do is comparable to that of the human newborn (Siegrist, 2001; Siegrist, 2007). First, the immunogenicity of the DNA vaccines was evaluated by determining the frequency of peptide-specific splenic IFN-γ-producing cells using the ELISPOT assay. For that purpose, splenic cells from mice immunized during the neonatal period were stimulated with pools of HIV-1-Gag peptides (25 pools spanning the entire Gag protein). We also used conventional ELISA to measure anti-Gag Ab in serum from DNA-vaccinated mice.

Fig. 1A shows that splenic cells obtained from neonatal mice primed with a relatively low dose (1 μg) of *LAMP*/*gag* DNA vaccine and followed by a boost with *gag* DNA vaccine secreted IFN-γ upon stimulation with Gag peptides in 18 (72%) of 25 pools. Two doses of *LAMP*/*gag* (1 μg) promoted a T cell response in 11 peptide pools (44%), while only 6 pools of peptides (24%) were recognized by neonates immunized with two doses of *gag* (1 μg) vaccine (Fig. 1B). The prime–boost schedule *LAMP*/*gag*+*gag* led to a significantly higher number of IFN-γ-secreting cells in three peptide pools compared to *gag* immunization, whereas those that received two doses of *LAMP*/*gag* (1 μg) differed by a single peptide pool. In the next protocol (Fig. 1C), we asked whether increasing the dose of the DNA vaccine (to 5 μg) would increase the amplitude of Gag peptide recognition. To test this, we evaluated only *LAMP*/*gag* and *gag* two-dose schedules (LG+LG and G+G). The 5-μg DNA immunization induced higher numbers of IFN-γ-secreting cells in mice vaccinated with two doses of *LAMP*/*gag*, generating a significant increase for eight peptide pools when compared to *gag* immunization (Fig. 1C). Increasing the chimeric DNA vaccine dose of 1 μg to 5 μg promoted a wider amplitude of peptide recognition. The main Gag peptides recognized by class I and II MHC molecules after adult *LAMP*/*gag* and *gag* immunization were located in peptide pools containing aa 181–227 (for MHC class I) or aa 241–271 and 281–311 (for MHC class II) based on previous findings (de Arruda et al., 2006). Thus, *LAMP*/*gag* vaccination elicited greater numbers of IFN-γ-secreting cells in the aa 181–211 pool containing the immunodominant class I-restricted epitope AMQMLKETINAAEEA when compared to *gag* vaccination. Interestingly, although the adult mouse immunization schedule was 50 μg per dose, the neonatal *LAMP*/*gag* immunization with 5 μg seemed to elicit immune responses of equal or greater magnitude with some peptide pools (Fig. 1D). This finding showed that neonatal immunization with the chimeric DNA vaccine evoked a stronger and wider cellular IFN-γ immune response to Gag peptides than did *gag* vaccination alone and that neonatal chimeric DNA immunization generated a similar response to adult immunization.

When we assessed the anti-Gag antibody responses elicited by the neonatal DNA immunization protocols (with 1 or 5 μg of DNA) and those of adult-immunized mice (50 μg of DNA), we found that priming with *LAMP*/*gag* (1 μg) followed by a *gag* boost was more efficient at eliciting anti-Gag IgG (Fig. 2A), composed of IgG2a and IgG2b, as compared to immunization with two *LAMP*/*gag* (1 μg) doses, which essentially induced anti-Gag IgG1 (Figs. 2A–C). In neonatal mice immunized with 5 μg of *LAMP*/*gag*, the subclass antibody response was similar to adult *LAMP*/*gag* DNA-immunized mice, inducing anti-Gag IgG1, IgG2a and IgG2b. However, immunization with *gag* (50 μg) elicited only anti-Gag IgG at low levels in adult mice (Figs. 2A and B). Control neonate and adult mice injected with plasmid containing only the *LAMP-1* gene (L+L) did not generate either IFN-γ-secreting cells recognizing Gag peptides (data not shown) or specific Ab.

Taken together, these findings show that with low DNA amounts, the *LAMP*/*gag*—*gag* prime–boost schedule was sufficient to generate a comparable immune response to the protocol of two 5-μg doses of *LAMP*/*gag*. Furthermore, chimeric *LAMP*/*gag* DNA was better than conventional *gag* DNA at eliciting cellular and humoral responses following neonatal immunization.

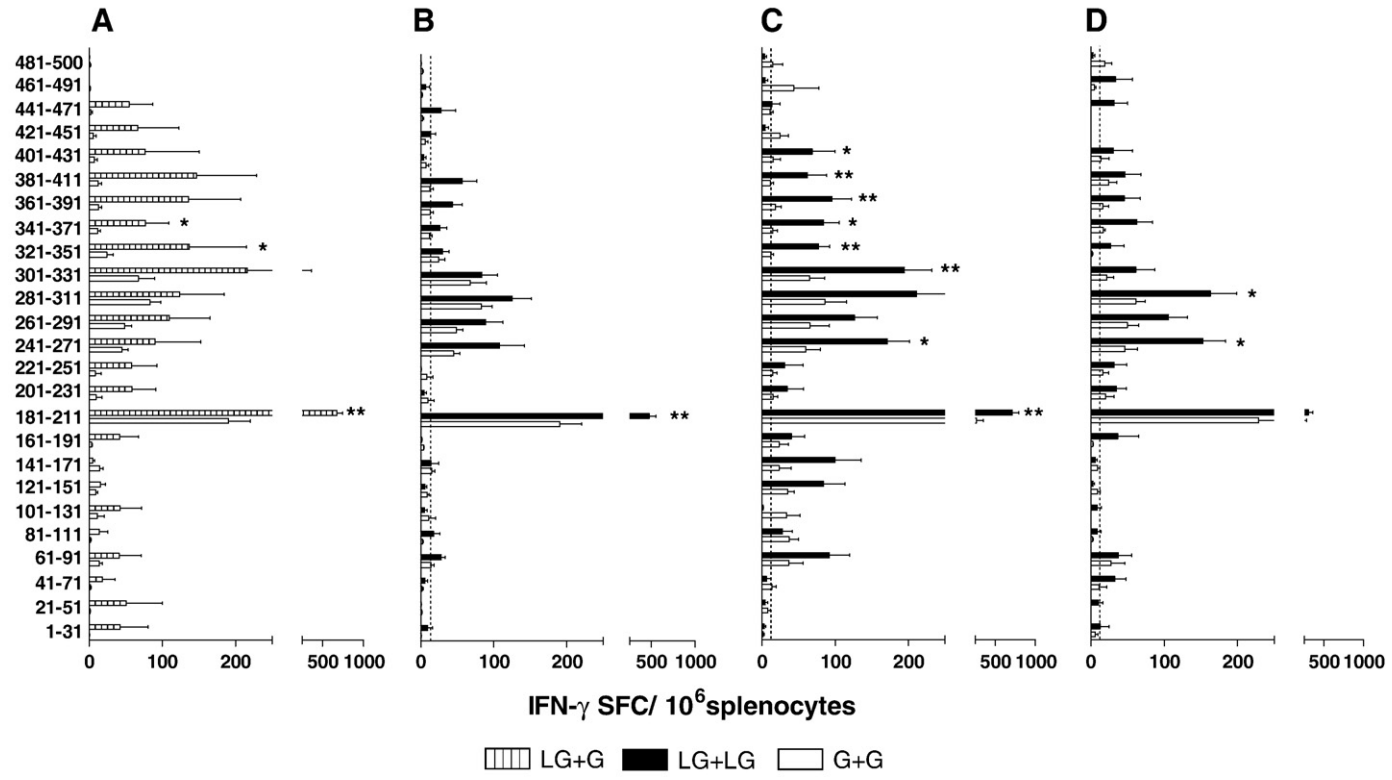

Fig. 1. Broad Gag-specific IFN-γ responses in young mice after neonatal immunization with *LAMP/gag* DNA vaccine. Neonatal BALB/c mice were primed at 7 days old (do) and boosted at 25 do with *LAMP/gag* or native *gag* DNA vaccine. Adult mice were immunized with 50 μg of DNA twice at 20-day intervals. Ten days after the last dose, the number of IFN-γ-secreting splenocytes was determined by ELISPOT assay upon stimulation with 123 peptides grouped in 25 pools containing 5 peptides, each encompassing the complete p55Gag protein sequence (15-aa peptides overlapping by 11 residues) (represented by the *Y* axis: 1–500 aa). (A) One-microgram neonatal prime/boost with *LAMP/gag* plus *gag* (LG + G) or *gag* plus *gag* (G + G). (B) One-microgram neonatal prime/boost with only *LAMP/gag* (LG + LG) or *gag* (G + G); (C) five-microgram neonatal prime/boost with *LAMP/gag* or *gag*; (D) adult prime/boost with 50 μg of *LAMP/gag* (LG + LG) or *gag* (G + G). The results presented are the mean ± SEM of 4–5 experiments, each comprising a pool of spleen cells (2–3 mice/group). Spontaneous spot-forming cells (SFCs) were less than 4 IFN-γ SFC/10^6; this value was subtracted from the stimulated condition and is represented on the *X* axis, and the peptide pools are represented by the ordinal Gag protein amino acid numbers (1–500 aa) on the *Y* axis. A response was considered as positive when the SFC number was higher than 10 IFN-γ SFC/10^6 cells. $^*P \leq 0.05$, $^{**}P \leq 0.01$ when compared with *gag*-immunized mice.

Enhanced T cell responses to Gag H-2^d class I and class II-restricted peptides after neonatal LAMP/gag immunization

Next, we analyzed cytokine production by CD4+ and CD8+ T cells upon stimulation with Gag H-2^d class I or class II-restricted immunodominant peptides or with p24Gag. The pools containing the 181–227 aa peptide (MHC class I-restricted epitope) and the 241–271 aa and 281–311 aa peptides (MHC class II-restricted epitope) were recognized when CD4+ or CD8+ T cells were depleted, respectively (Arruda et al., 2006).

Analyzing the protocol in which neonate mice were immunized with the low dose of DNA (1 μg), we detected a higher number of IFN-γ secreting cells for the class I-restricted immunodominant epitope in animals that received one dose of *LAMP/gag* followed by *gag* or two

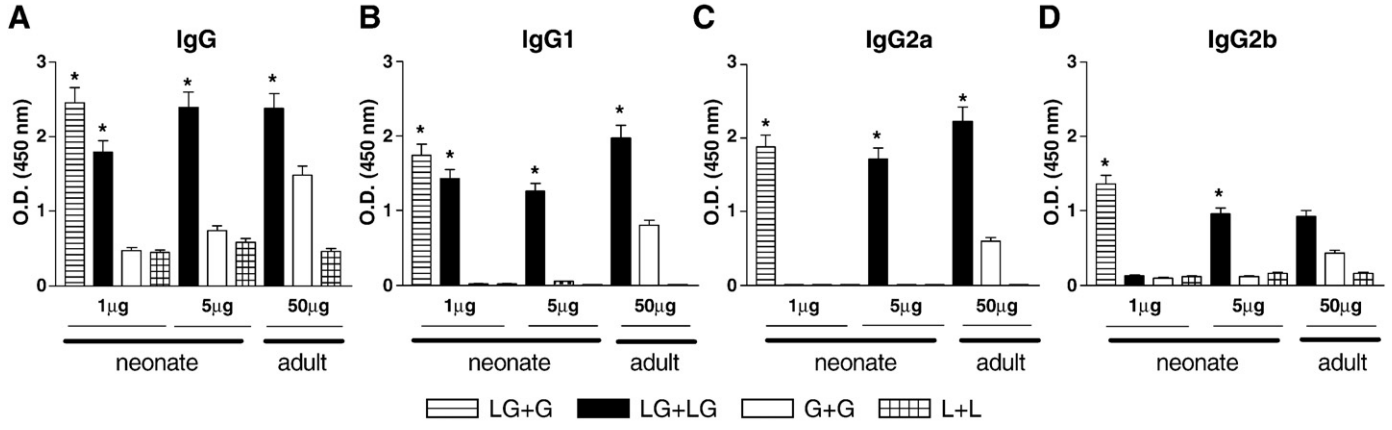

Fig. 2. Neonatal priming with *LAMP/gag* DNA vaccine is essential for eliciting anti-Gag IgG antibodies. Neonates were primed at 7 do and boosted at 25 do with 1 and 5 μg of plasmid DNA according to the following the prime–boost schedules: *LAMP/gag* + *gag* (LG + G); *LAMP/gag* + *LAMP/gag* (LG + LG); *gag* + *gag* (G + G) and *LAMP* + *LAMP* (L + L). Adult mice were immunized twice with 50 μg of DNA vaccine at 20-day intervals. Anti-Gag antibody levels of IgG (A), IgG1 (B), IgG2a (C) and IgG2b (D) subclasses were evaluated in serum pools (three pools of nine mice/group, neonate serum dilution, 1:900; adults, 1:2700) ten days after the boost by ELISA. Each bar represents the mean ± SEM. $^*P \leq 0.05$ when compared with *gag*-immunized mice.

LAMP/*gag* doses than in those immunized only with *gag* (Fig. 3A). These increases were much greater when higher vaccine doses were used (Fig. 3D). In addition, higher secretion levels of IFN-γ upon class I-restricted peptide stimulation were detected in the mice immunized with *LAMP*/*gag* + *gag*, and TNF-α was higher when the mice received two doses of *LAMP*/*gag* compared to *gag* alone (Figs. 3B and C, respectively), this similar profile of cytokine production was also observed in the neonatal immunization protocol using the higher dose of DNA (5 μg), (data not shown). We then analyzed the number of IFN-γ-secreting cells from neonatal immunized mice upon stimulation with Gag H-2^d class I or class II-restricted immunodominant peptides or with p24Gag. Vaccination with 5 μg of *LAMP*/*gag* DNA promoted a higher number of IFN-γ-secreting cells recognizing Gag H-2^d class I or class II-restricted immunodominant peptides and p24Gag than did vaccination with *gag* DNA alone (Figs. 3D–F). These results show that *LAMP*/*gag* immunization generated more Gag-specific CD8+ and CD4+ T cells compared to *gag* immunization alone.

Next, we assessed the immune responses to neonatal and adult immunization using two 5-μg doses of chimeric *LAMP*/*gag* by evaluating the frequency of IFN-γ-secreting cells recognizing H-2^d Gag class I and II-restricted peptides and p24 Gag. Unexpectedly, neonatal immunization elicited significantly higher numbers of IFN-γ-secreting cells recognizing Gag MHC class I-restricted peptide and p24 than did adult immunization (Fig. 3G). For the class II peptides, a similar frequency of IFN-γ-secreting cells was verified in both groups of mice. This similarity was only related to *LAMP*/*gag* vaccination, because *gag* immunization induced similar IFN-γ responses in neonatal and adult immunization (data not shown).

Increased CD8+ T and CD4+ T cell function following neonatal immunization with chimeric vaccine

To evaluate the phenotypic and functional characteristics of immunodominant CD8+ T cells, we analyzed only the neonatal immunization protocols using the higher DNA dose (5 μg), because this dose elicited a response to Gag peptides by IFN-γ-secreting cells (Fig. 1C) and production of anti-Gag antibody (Fig. 2). We also included a third neonatal immunization protocol (5 μg) that consisted

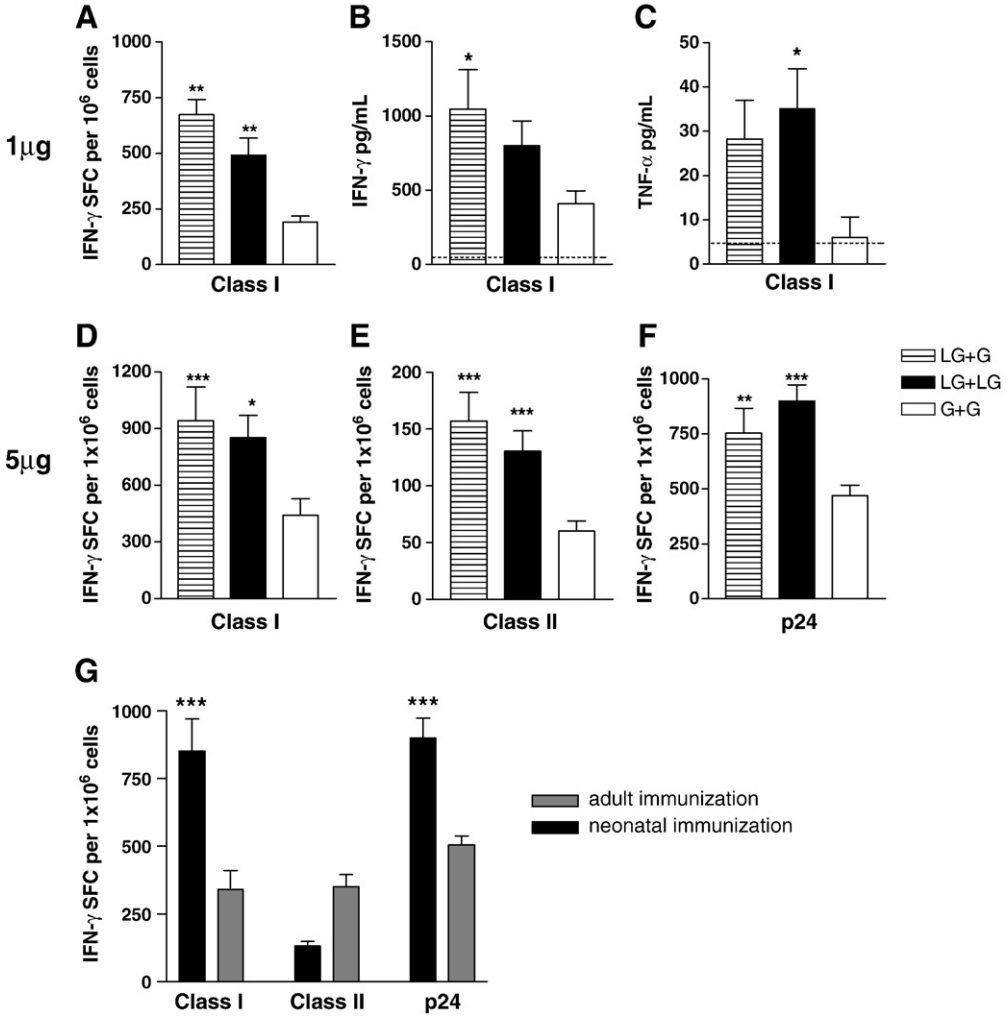

Fig. 3. Neonatal priming with *LAMP*/*gag* DNA vaccine induced robust CD8+ and CD4+ T cell responses. Neonates were immunized with 1 or 5 μg in the following prime–boost schedules: *LAMP*/*gag* + *gag* (LG + G, striped bar), *LAMP*/*gag* + *LAMP*/*gag* (LG + LG, closed bar) and *gag* + *gag* (G + G, open bar). Adult mice were immunized with 5 μg of the prime–boost schedule *LAMP*/*gag* + *LAMP*/*gag* (LG + LG). (A, D) Splenic IFN-γ-secreting cells recognizing the immunodominant Gag MHC class I-restricted H-2K^d peptide (AMQMLKETINAAEE) were evaluated by ELISPOT (5–8 mice/group). (B) IFN-γ and (C) TNF-α production by splenocytes from neonatally immunized mice (n = 5–6) cultured for 72 h with 10 μg/mL immunodominant Gag MHC class I-restricted H-2K^d peptide and evaluated by flow cytometry using a cytometric bead array. The results represent the difference between the cytokine levels produced after stimulation and spontaneous production. The dashed line represents the assay detection limit. (D, E, F) Splenic IFN-γ-secreting cells recognizing the immunodominant Gag MHC class I and II-restricted H-2^d peptides and Gag p24 were evaluated by ELISPOT (5–8 mice/group). (G) Splenic IFN-γ-secreting cells of neonate (closed bar) or adult (gray bar) immunized with 5 μg of DNA (LG + LG). Each bar represents the mean ± SEM of 4–5 experiments. $^*P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P < 0.001$ when compared with *gag*-immunized mice , $^{***}P < 0.001$ when compared to adult immunization.

of priming with *LAMP/gag* and boosting with *gag*. The number of Gag-specific CD8+ T cells binding the H-2K^d pentamer bearing the MHC class I-restricted Gag epitope was estimated following immunization. Fig. 4A shows that neonatal mice immunized with *LAMP/gag+gag* had a significantly higher frequency of Gag epitope-specific CD8+ T cells (~2.8%) compared to mice immunized with two doses of *LAMP/gag* (~1.8%) or *gag* alone (~1.7%). Moreover, the *in vivo* CTL response to the Gag H-2K^d MHC class I-restricted epitope revealed that the vaccination schedules with *LAMP/gag+gag* and *LAMP/gag+LAMP/gag* improved CD8+ T cell cytotoxic function compared to *gag* immunization (Fig. 4B).

The higher CTL response after neonatal *LAMP/gag+gag* immunization correlated with higher percentages of CD8+ T cells expressing LAMP-1 (CD107a) and Granzyme B+ (Fig. 4C). In addition, the greater frequency of Gag epitope-specific CD8+ T cells in *LAMP/gag+gag* mice than in *gag+gag* mice was related to the significantly higher number of CD8+IFN-γ+ (Fig. 4D) and CD8+TNF-α+ cells (Fig. 4E). Despite the fact that neonatal immunization with conventional *gag* DNA generated similar percentages of Gag epitope-specific CD8+ T cells, these cells had no CTL activity compared to those generated by neonatal *LAMP/gag* priming (4B).

Because the LAMP/gag chimeric protein is targeted to class II MHC-rich cell compartments (Marques et al., 2003), we assessed the CD4+ T cell proliferative response to p24Gag as well as the CD8+ T cell response to class I-restricted immunodominant Gag peptide by [^{3}H] thymidine uptake.

To determine which cell population was induced by the neonatal *LAMP/gag* and *gag* DNA immunizations, we evaluated these schedules using two doses of *LAMP/gag* or *gag*. Higher proliferative responses to MHC class II-restricted Gag peptide and p24Gag were observed in splenic cells from mice neonatally immunized with *LAMP/gag* than in cells from *gag*-immunized mice (Fig. 5A), suggesting greater CD4+ T cell stimulation. In addition, neonatal immunization with *LAMP/gag* generated higher percentages of CD4+ T cells that proliferated in response to p24 than did neonatal *gag* immunization (Fig. 5B), suggesting enhanced CD4+ T cell activation. Neonatal chimeric DNA vaccination also enhanced the CD8+ T cell proliferative response to p24Gag protein (Fig. 5C) as well as to the immunodominant Gag H-2^d MHC class I-restricted epitope (Fig. 5D).

These results show that priming the neonatal immune system with *LAMP/gag* chimeric DNA vaccine was more effective at generating Ag-specific CTLs and at inducing the proliferation of CD4+ T and CD8+ T cells than was the conventional *gag* DNA vaccine.

Long-lasting cellular and humoral responses following neonatal LAMP/gag vaccination

We next determined whether only neonatal priming with *LAMP/gag* vaccine could induce long-lasting effector cells that secrete IFN-γ after Gag peptide stimulation. First, neonatal mice were primed with 5 μg of the DNA vaccine at 7 do and boosted at adult age (six months old) with 5 μg of the same DNA vaccine. This protocol was used to assess whether only the neonatal priming with *LAMP/gag* at seven days old without the boost at 25 days old could induce more IFN-γ-secreting cells at six months of age than conventional *gag* immunization.

The results clearly showed that *LAMP/gag* priming during the neonatal period led to a higher frequency of IFN-γ-secreting cells recognizing ten pools of Gag peptides, including MHC class I and II-restricted peptides, than did conventional *gag DNA* immunization (Fig. 6A). In addition, only the mice that were primed during the neonatal period with *LAMP/gag* developed high levels of anti-Gag IgG (Fig. 6B). To verify the importance of the priming during neonatal

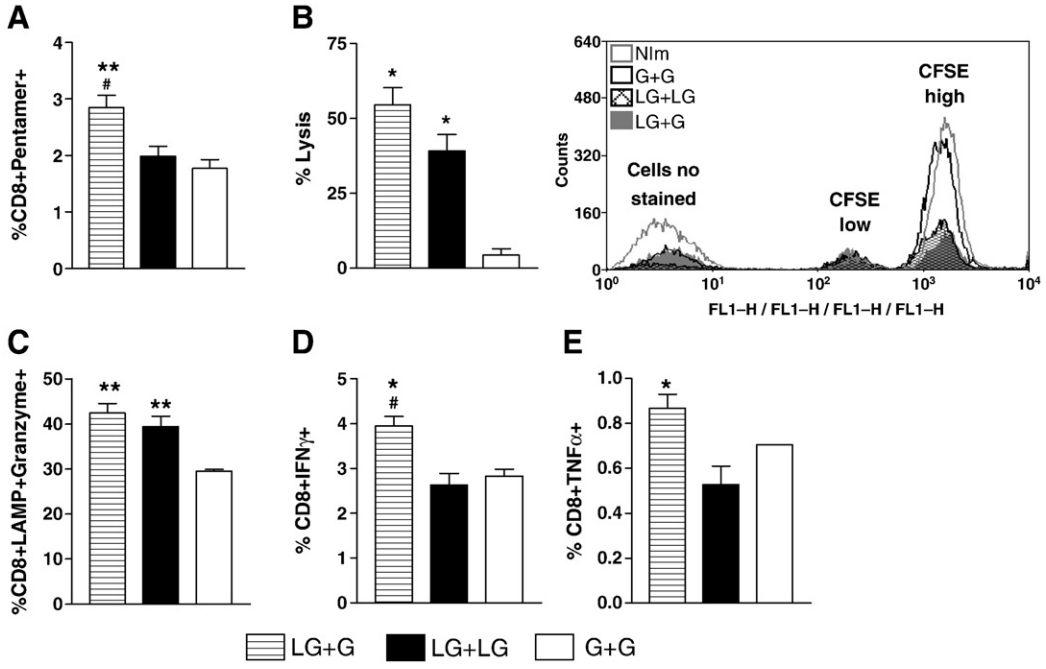

Fig. 4. Characterization of the CD8+ T cell response to neonatal *LAMP/gag* vaccination. Neonatal mice were immunized with 5 μg in the following prime–boost schedules: *LAMP/gag+gag* (LG+G, striped bar), *LAMP/gag+LAMP/gag* (LG+LG, closed bar) and *gag+gag* (G+G, open bar). Ten days later, the mice were evaluated. (A) Ex-vivo percentage of CD8+pentamer+ cells for the H-2K^d class I immunodominant Gag peptide. (B) *In vivo* T cell cytotoxicity was evaluated after the neonatal prime–boost DNA immunization protocols. Ten days after the second dose of DNA vaccines, immunized and non-immunized mice were iv-injected with target cells from non-immune mice stained with low and high-CFSE concentrations. The high-CFSE target cells were pulsed with class I immunodominant peptide (AMQMLKETI). After 18 h, splenic cells from immunized and non-immunized (Nlm) mice were evaluated to detect the percentage of lysis. The histogram shows the diminished high-CFSE population in neonatally immunized LG+G and LG+LG mice compared to G+G mice. (C) *Ex vivo* intracellular expression of granzyme and extracellular expression of LAMP-1 on CD8+ T cells by flow cytometry. Splenocytes were incubated with the immunodominant Gag H-2K^d MHC class I-restricted peptide overnight for detection of (D) IFN-γ and (E) TNF-α expression in CD8+ T cells by flow cytometry. $^*P \leq 0.05$, $^{**}P \leq 0.01$ when compared to *gag*; and $\#P \leq 0.05$ when compared to mice immunized with two doses of *LAMP/gag*.

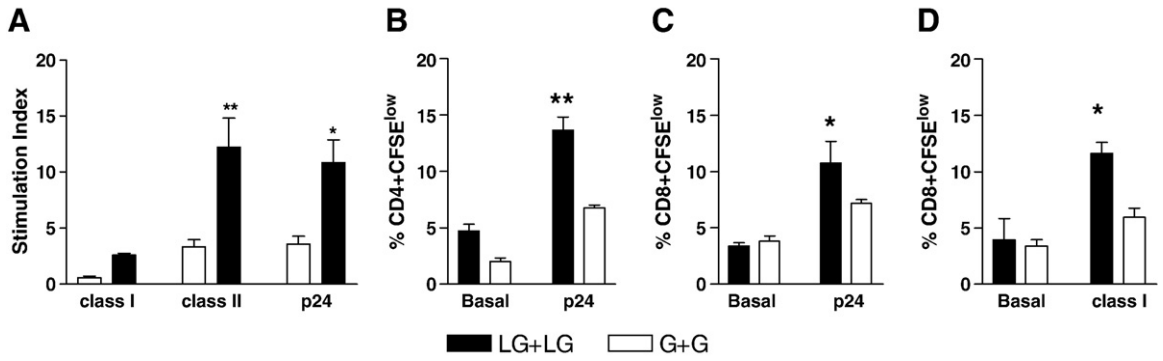

Fig. 5. Neonatal *LAMP/gag* DNA immunization induced greater proliferation of CD8+ and CD4+ T cells than conventional *gag* DNA immunization. (A) Splenic cells from mice neonatally immunized with 5 μg in the prime–boost schedules *LAMP/gag* + *LAMP/gag* (LG + LG) and *gag* + *gag* (G + G) were cultured with Gag MHC class I and II-restricted H-2^d peptides and p24 from HIV-1 for seven days. [^{3}H]thymidine uptake is presented as the stimulation index (SI). (B, C, D) CD4+ and CD8+ proliferation measured by CFSE. Splenic cells stained with 5 μM CFSE were stimulated with (B, C) 2 μg of HIV p24 or (D) 10 μg of Gag MHC class I H-2^d-restricted peptide for six days. CD4+, CD8+ and low-CFSE cells were analyzed by flow cytometry. $^*P<0.05$ or $^{**}P<0.05$ when compared with *gag*.

period, at seven days old, we performed a control experiment in which groups of mice received priming only (one vaccine dose) at six months of age. These mice developed fewer IFN-γ-secreting cells recognizing the Gag MHC class I and II-restricted peptides than did mice primed at 7 do (Fig. 6C). These results further indicated that neonatal priming with the chimeric DNA efficiently generated IFN-γ effector memory cells.

To verify the activated/memory phenotype of the CD4+ and CD8+ T cells after neonatal immunization (at 7 and 25 do), we analyzed the expression of activation and memory markers and cytokines on splenocytes in two situations: *ex vivo* or after stimulation with Gag H-2^d class I or II-restricted peptides. We evaluated the expression of CD107a, a marker for cytolytic activity (LAMP-1), CD127 (an IL-7 receptor expressed in memory T cells) and CD69 (early activation). At six months of age, mice immunized as neonates with *LAMP/gag* and boosted with *gag* showed higher *ex vivo* percentages of CD8+ T cells co-expressing CD107a and granzyme than did mice immunized with *gag* (Fig. 7A), and upon stimulation with the Gag H-2^d class I-restricted epitope, they showed a slightly higher percentage of CD8+ cells expressing CD69+ (Fig. 7B), indicating a higher percentage of CD8+ T cells with an early activated

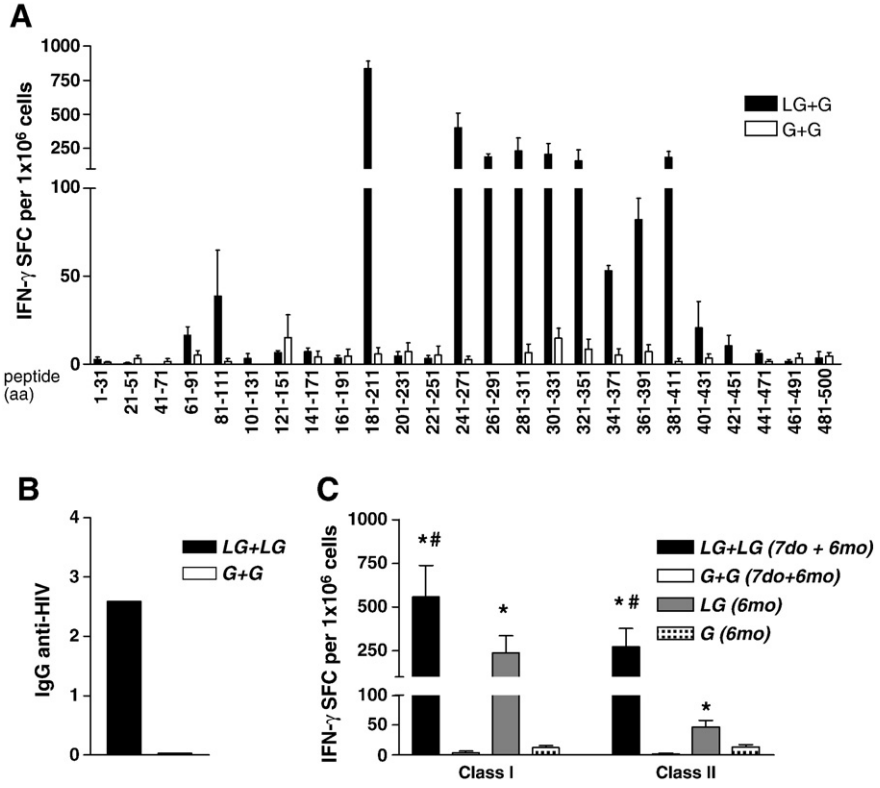

Fig. 6. Long-lasting T cell and humoral responses after neonatal priming with chimeric *LAMP/gag* DNA. Neonatal mice were primed with 5 μg of DNA vaccine at 7 do and boosted after six months with 25 μg in the following prime–boost schedules: *LAMP/gag* + *LAMP/gag* (LG + LG, closed bar) and *gag* + *gag* (G + G, open bar). (A) Ten days after the boost, splenic IFN-γ-secreting cells recognizing 25 pools of Gag peptides were evaluated by ELISPOT. The results presented are the mean ± SEM of three experiments, each using a pool of splenic cells (three mice/group). (B) Anti-Gag IgG (1:900) was determined in serum in six-month-old mice neonatally immunized mice by ELISA. (C) IFN-γ-secreting cells recognizing Gag H-2^d MHC class I and II-restricted epitopes from six-month-old mice neonatally immunized at 7 do and from mice immunized only at 6 months. $^*P \leq 0.05$, when compared with gag #$P \leq 0.05$ when compared with mice immunized at 6 months.

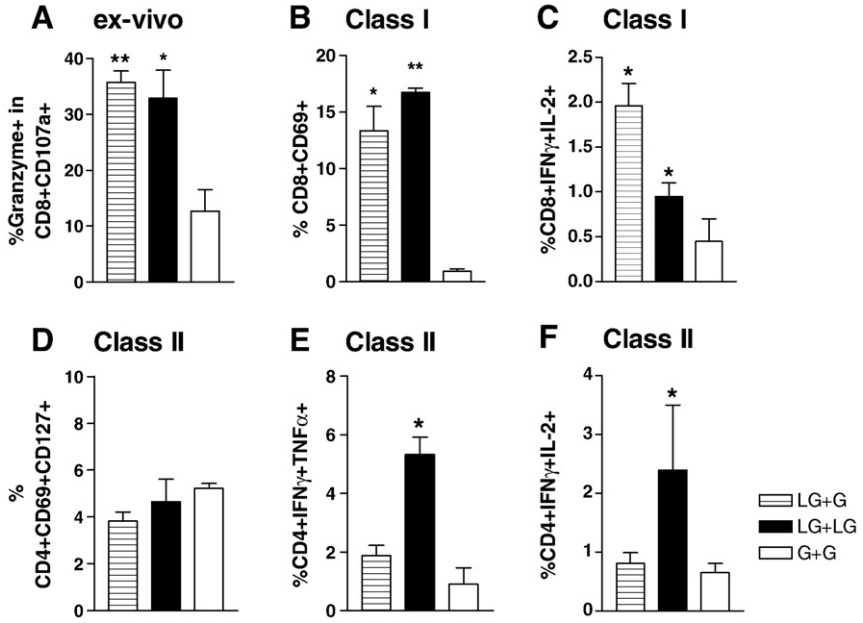

Fig. 7. Phenotypic features of long-lasting T cell responses elicited by neonatal immunization with chimeric *LAMP/gag* DNA. Neonates were primed with 5 μg of DNA vaccine at 7 and 25 do in the following prime–boost schedules: *LAMP/gag* + *gag* (LG + G), *LAMP/gag* + *LAMP/gag* (LG + LG) and *gag* + *gag* (G + G), and spleen cells were analyzed after six months. Splenocytes were evaluated *ex vivo* to detect (A) intracellular expression of granzyme in CD8+CD107a+ cells. Splenocytes were evaluated upon stimulation with Gag H-2^d MHC class I or II-restricted peptides for six hours to detect extracellular expression of (B) CD8+CD69+; (D) CD4+CD69+CD127+ and to detect intracellular expression of (C) IFN-γ/IL-2 in CD8+ (F) and CD4+ T cells and (E) IFNγ/TNFα CD4+ T cells by four-color flow cytometry. The results represent the mean ± SEM of 3–4 animals. $^*P \leq 0.05$, $^{**}P < 0.01$ when compared to *gag*-immunized mice.

phenotype. Moreover, we assessed the polyfunctional ability of the CD8+ and CD4+ T cells to express intracellular IFN-γ/IL-2 (an indicator of proliferation and activation) and IFN-γ/TNF-α (an indicator of cytotoxicity and activation). The immunological memory response to the Gag MHC class I-restricted peptide revealed higher percentages of CD8+ T cells co-expressing IFN-γ/IL-2 in mice immunized with the chimeric DNA vaccine than in those immunized with conventional *gag*, 1.9% versus 0.70% (Fig. 7C). No change in the percentage of CD4+ cells expressing CD69 was detected in stimulated splenic cells after neonatal immunization (Fig. 7D). However, two doses of *LAMP/gag* generated slightly higher percentages of CD4+ T cells co-expressing IFN-γ/TNF-α (5.3% versus 0.5%, Fig. 7E) and IFN-γ/IL-2 (2.4% versus 0.6%, Fig. 7F) after stimulation with Gag MHC class II-restricted peptide than did neonatal *gag* immunization. These findings clearly indicate that when neonatal mice were immunized with *LAMP/gag* + *gag*, only augmented levels of the CD8+ T cell response were achieved, but when neonates were immunized only with chimeric vaccine (*LAMP/gag* + *LAMP/gag*), long-lasting CD4+ and CD8+ effector T cells were generated.

Fig. 8A shows that up to nine months after *LAMP/gag* neonatal vaccination, broadly higher numbers of IFN-γ-secreting cells recognizing Gag peptide pools as well as Gag MHC class I and II-restricted peptides were detected, as compared to after conventional *gag* immunization. Curiously, the schedule using two doses of *LAMP/gag* vaccine resulted in the recognition of three peptide pools, while the immunization protocol with one dose of *LAMP/gag* followed by *gag* recognized only one. It was remarkable that the Ab levels detected in nine-month-old mice immunized with chimeric vaccine did not require a boost for maintenance (Fig. 8B). In contrast to the findings with IFN-γ-secreting cells recognizing Gag, the most efficient production of anti-Gag IgG Ab was in the group receiving the *LAMP/gag* + *gag* schedule

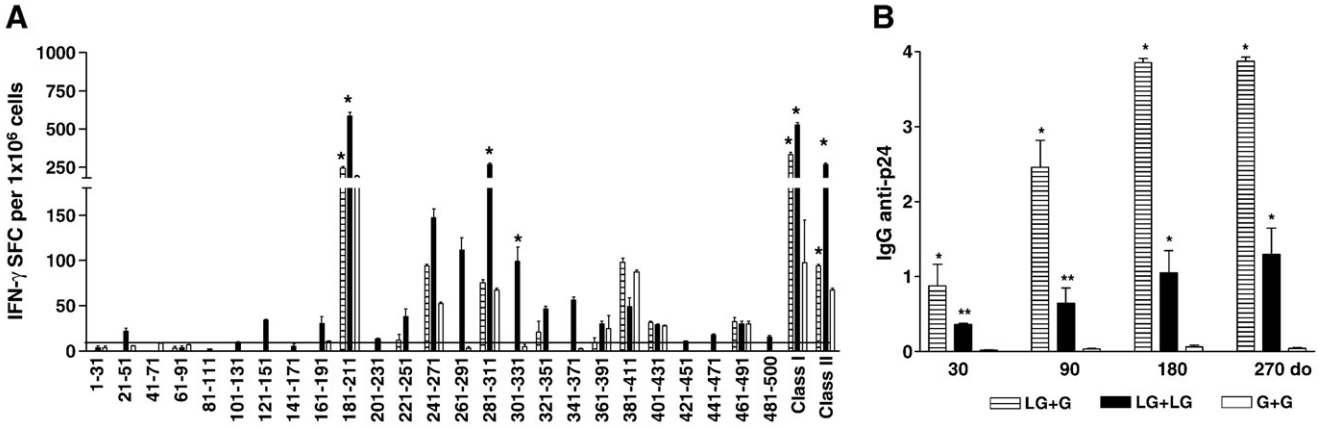

Fig. 8. Duration of Gag immune responses generated after neonatal immunization using chimeric *LAMP/gag* DNA. Neonatal mice were immunized with 1 or 5 μg in the following prime–boost schedules: *LAMP/gag* + *gag* (LG + G), *LAMP/gag* + *LAMP/gag* (LG + LG) and *gag* + *gag* (G + G). (A) At nine months of age, IFN-γ-secreting cells recognizing the 25 pools of Gag peptides were evaluated by ELISPOT. The results presented are the mean ± SEM of three experiments, each using a pool of splenic cells (three mice/group). (B) IgG anti-Gag (1:900) was determined in serum at different ages up to nine months of age (270 do) by ELISA. $^*P \leq 0.05$, $^{**}P \leq 0.01$ when compared to *gag*.

(Fig. 8B). Mice immunized with *gag* and a control *LAMP* plasmid (data not shown) produced no detectable anti-p24 IgG Ab at nine months. These findings reinforce the concept that targeting the antigen to the class II MHC compartment during neonatal vaccination is better at generating immunological memory and long-lasting T and B effector functions.

Discussion

The main finding of our study is that for a DNA vaccine to be immunogenic enough to provide long-lasting responses after neonatal immunization in mice, it is important to target the antigen to compartments containing MHC class II molecules. We achieved this goal by comparing a chimeric *LAMP*/*gag* DNA vaccine to a conventional *gag* DNA vaccine. Neonatal immunization using *LAMP*/*gag* elicited broad recognition of HIV-1 Gag MHC class I and II-restricted epitopes and induced a robust CTL response and CD4+ T cell proliferation favoring anti-Gag Ab production. These results emphasize the concept that when the neonatal immune system is adequately stimulated by a vaccine formulation, it is able to establish an Ag-specific IFN-γ response similar in magnitude to that of adult-immunized mice. Targeting the HIV Gag protein to MIIC compartments in the neonatal period contributes to the development of improved immune responses that will last throughout adult life.

The administration of a relatively low dose of *LAMP*/*gag* (1 μg) in BALB/c neonates was able to significantly increase the frequency of IFN-γ-secreting cells recognizing the immunodominant Gag MHC class I-restricted H-2^d epitope AMQMLKETI$_{197–206}$ as well as the secretion of IFN-γ and TNF-α. These cytokines may be involved in the anti-HIV CTL response (Lichterfeld et al., 2004). In fact, we detected improved levels of *in vivo* CTL responses against Gag in mice immunized as neonates with one or two doses of *LAMP*/*gag*. Therefore, the chimeric vaccine promoted broad recognition of immunodominant Gag MHC class I and II-restricted epitopes following neonatal immunization. These findings emphasize the flexibility of the neonatal immunological response, which, when adequately stimulated, can induce antigen-specific cytotoxic T cells, probably due to competent antigen processing and presentation by MHC class I molecules of APCs (Gold et al., 2007). The doses administered in the neonatal DNA immunization protocols were relatively low compared to other murine neonatal DNA immunization protocols (Zhang et al., 2002; Martinez et al., 1997; Sedegah et al., 2003). Considering the high propensity for tolerance in the neonatal period, the low doses used for neonatal immunization may overcome the tolerogenic effect and evoke an efficient cellular and humoral immune response. One immunization protocol that improved immunogenicity at the early stage of life was priming with *LAMP*/*gag* followed by a boost with *gag*, revealing the importance of *LAMP*/*gag* in a priming vaccination scheme, as already demonstrated in adult-immunized in mice (Arruda et al., 2004). The improvement of the immune response using the *LAMP*/*gag* plus *gag* neonatal immunization protocol was likely due to the generation of distinct and diverse MHC class I and II-restricted epitopes, which enabled stronger anti-Gag responses by CD4+ and CD8+ T cells. In fact, the profile of anti-Gag IgG subclasses was composed of IgG1 and IgG2a in mice immunized with *LAMP*/*gag*+*gag* (1 μg), showing activation of both Th2 and Th1 cell subtypes. This protocol enhanced humoral and cellular responses to HIV, as already demonstrated after adult immunization (Arruda et al., 2004). On the other hand, two doses of *LAMP*/*gag* (1 μg) triggered only IgG1, and an increase in the vaccine amount to 5 μg was required to produce a profile similar to the adult counterpart.

Furthermore, the immunization protocols with chimeric *LAMP*/*gag* vaccine were unique in promoting high anti-Gag IgG Ab levels. These results strengthen the concept of broad CD4+ T cell activation with *LAMP*/*gag* neonatal immunization, as verified by higher CD4+ T cell proliferation in response to Gag protein (p24) and to MHC class II Gag epitopes, as well as by increased proinflammatory cytokine expression (CD4+IFN-γ+IL-2+). The mechanism by which this *LAMP*/*gag* chimeric DNA vaccine elicits both humoral and cellular responses to the HIV Gag protein could have an important role in protecting the host from HIV infection. The augmented cellular response against Gag, one of the first antigens exposed on HIV-1-infected cell membranes (Sacha et al., 2007), is considered as a good prognostic indicator for AIDS evolution in HIV-1-infected children (Buseyne et al., 2002). It is possible that HIV candidate vaccines that induce an immune response against this early-expressed and conserved HIV protein, even before the viral cycle has finished (Sacha et al., 2007), could represent important tools for eliminating the cells infected early and, thereby, could inhibit the spread of virus.

Interestingly, immunizing neonatal mice with *LAMP*/*gag* induced a higher frequency of IFN-γ-secreting cells in response to p24 and H-2^d Gag class I-restricted peptide pools than did immunizing adult mice with the same dose of DNA. It is possible that T cells from the antigen-inexperienced microenvironment found in the postnatal phase can be more effectively primed than those from the experienced immune system, even in specific pathogen-free conditions. It seems that in the neonatal period, the trafficking of the chimeric *LAMP*/*gag* protein through the MIIC compartment rich in MHC class II molecules was important for promoting epitope recognition by CD4+ T cells and, consequently, for generating humoral and cellular responses, as reported in adult mice (Marques et al., 2003; de Arruda et al., 2004). The strategy of the *LAMP*/*gag* construct made it possible to overcome the inadequate expression of MHC class II and costimulatory molecules on APCs in the neonatal period (Min et al., 2001), which are typically requirements for the generation of an efficient adaptive immune response. Several studies in mice and humans showed that the primary T cell-dependent Ab responses induced in the neonatal period differ from adult responses (Sigriest, 2001; Adkins et al., 2004; Rigato et al., 2009). On the other hand, in our study, the cellular Ag-specific IFN-γ response was comparable between neonatal and adult immunization, while Ab production following neonatal *LAMP*/*gag* immunization was lower than that following adult immunization. The lower Ab production in neonates could be related to neonatal immune characteristics, such as the high proportion of immature splenic B cells (IgM IgD$^{low/-}$) and negative BCR signaling (Adkins et al., 2004). Nevertheless, the *LAMP*/*gag* vaccine was essential for generating a B cell memory response, as verified by the long-lasting humoral response observed up to nine months of age.

The chimeric vaccine was unique in priming at the neonatal phase, it led to efficient CD4+ T cell activation (an essential mechanism for generating long-lasting CD8+ T and CD4+ T effector cells), and at six months, it led to double-positive intracellular cytokine expression. For an anti-HIV vaccine that generates strong cellular immune effectors, such as cytokines, antibodies or CTL generation, avoiding an excess of immune activation could be optimal for clearing the infection. Although significant increases in CD4+ T cell function (as assessed by cytokine production and proliferation) and CD8+ effector T cells followed chimeric DNA immunization, regulatory mechanisms could also be triggered by the vaccination, and these should be evaluated in future studies.

Long-lasting immunological memory at the B and T cell level was verified up to nine months after neonatal immunization. These results confirm the finding obtained with adult mice immunized with the chimeric DNA vaccine (de Arruda et al., 2004). This reinforces that memory CD4+ and CD8+ T cells may remain in the absence of antigen and proliferate to maintain immunological homeostasis (Amanna et al., 2006), despite the fact that the persistence of plasmid *in vivo* needs to be evaluated in this model. Residual plasmid DNA has been detected months after immunization (Coelho-Castelo et al., 2006). The persistence of antibodies after neonatal *LAMP*/*gag* immunization up to nine months of age could be due to the generation and maintenance of long-lived or short-lived plasma cells or to plasmid transcription in long-lived transfected cells.

The generation of long-lived plasma and memory T cells is dependent on CD4+ T cell activation; however, the maintenance of the plasma cells is independent of CD4+ T cells and antigen presence (Maecker et al., 1998; Dorner and Radbruch, 2007; Chan et al., 2001). The survival mechanisms of memory B cells and long-lived plasma cells have not yet been elucidated, but factors such as CXCL-12, IL-6, BAFF and CD44 seem to be required for, although not essential to, their survival and maintenance (Cassese et al., 2003). Mice infected with vesicular stomatitis virus revealed that long-lived plasma cells were responsible for the permanence of antibodies up to 300 days after infection (Maecker et al., 1998).

Until now, few DNA vaccine strategies that lack cytokines or adjuvants have been capable of generating and maintaining long-lasting cellular and Ab anti-HIV responses when used in neonatal mice. Further investigations addressing neonatal vaccination with *LAMP/gag* DNA vaccine for the maintenance of long-lived plasma cells and the role of these anti-Gag antibodies are required. Taken together, these results demonstrate that the chimeric *LAMP/gag* DNA vaccine is capable of promoting cellular and humoral anti-Gag responses in the neonatal period that can last until adulthood. Thus, *LAMP/gag* given at an early phase of life could establish an anti-HIV response that lasts until adolescence and sexual initiation, and thereby, it could prevent breastfeeding transmission. As a therapeutic strategy, *LAMP/gag* vaccine administered at an early phase of neonatal infection could improve the clearance of infected cells and might delay AIDS progression.

Materials and methods

Plasmids

Eukaryotic expression plasmids were constructed using nucleotides 1–1503 of the HIV-1 HXB2 *p55gag* gene (genBankTM accession number KO3455) inserted in the mammalian expression vector pITR (Kessler et al., 1996; Xin et al., 2003), which contains a cytomegalovirus promoter and ITR sequences from adeno-associated virus flanking the expression elements. The mouse *LAMP-1* gene (GenBank J03881) was also cloned into the same vector. For construction of the *LAMP/gag* plasmid chimera, the *p55gag* gene sequence was inserted between the luminal domain and the transmembrane/cytoplasmic tail of *LAMP-1*, as previously described (Marques et al., 2003; Chikhlikar et al., 2004, 2006; de Arruda et al., 2004; Arruda et al., 2006; Valentin et al., 2009). All plasmids were produced by transforming DH5α *Escherichia coli* (Invitrogen, Calsbad, CA) and purified using an endotoxin-free column (Qiagen Inc., Valencia, CA).

Mice immunization

BALB/c mice, aged 1 or 8–10 weeks old, were purchased from CEMIB (UNICAMP, Campinas, São Paulo, Brazil) and bred in our own specific pathogen-free laboratory's facilities. The São Paulo University Institutional Animal Care and Use Committee approved all animal studies.

Neonatal immunization

Seven-day-old mice of both sexes were immunized by the intradermal (i.d.) route with 1 or 5 μg of the DNA plasmids encoding either pITR/*LAMP/gag* (*LAMP/gag*), pITR/*gag* (*gag*) or pITR/*LAMP* (*LAMP*). The mice received a second dose at 25 days of age and were sacrificed ten days later. We used the intradermal route of immunization, which has previously been shown to elicit similar immunogenicity than when mice were immunized by intramuscular route (Arruda et al., 2004). Some groups were primed with *LAMP/gag* DNA and boosted with the *gag* DNA vaccine. To evaluate the long-term response to neonatal immunization, some groups of immunized neonates were followed for six or nine months and boosted with 25 μg of DNA vaccine ten days before sacrifice. Some mice were left untreated.

Adult immunization

Female adult mice (6–8 weeks old) received two intradermal doses of 50 μg or 5 μg of each DNA vaccine at 20-day intervals and were sacrificed 10 days later.

Antibody response

Anti-Gag IgG antibody levels were assessed by enzyme-linked immunosorbent assay (ELISA) as previously described (Marques et al., 2003). Briefly, 96-well microplates (Greiner, German) were coated with 5 μg/mL of HIVIIIB lysate (ABI, Rockville, MD) or 2 μg/mL of p24Gag (kindly provided by Prof. Luis Carlos Ferreira, Institute of Biomedical Sciences, University of São Paulo) and incubated overnight at 4 °C. After blocking with PBS + 0.5% gelatin for 1 h at 37 °C, the plates were washed three times with PBS and incubated with serial dilution samples for 2 h at 37 °C. After washes with 0.5% Tween in PBS, the plates were incubated with the biotinylated antibodies anti-γ, anti-γ1, anti-γ2a, anti-γ2b or anti-γ3 (SouthernBiotech, Birmingham, Alabama, USA) for 1 h at 37 °C. Streptavidin peroxidase (Sigma, St. Louis, MO, USA) was added for 1 h at 37 °C, and the reaction was revealed with tetramethyl benzidine (Calbiochem Corporation, San Diego, CA, USA). The absorbance was read at 450 nm in an ELISA microplate reader (Bio-Rad, USA).

Isolation of mononuclear spleen cells

Spleens were obtained aseptically and mashed through cell strainers (BD Bioscience, CA, USA). Mononuclear spleen cells (MSCs) were isolated after centrifugation on Ficoll–Hypaque solution (Sigma, St. Louis, MO, USA). After two washes, the final cell pellet was diluted in RPMI 1640 containing 10% fetal calf serum (FCS, HyCloneIII, Logan, UT, USA). Viability was greater than 95%.

Gag-specific IFN-γ ELISPOT assay

Enumeration of IFN-γ-producing splenic T cells was performed by ELISPOT assay according to the manufacturer's protocol (BD Biosciences Pharmingen, San Diego, CA, USA). Ninety-six-well microplates with polyvinylidene fluoride (PVDF) membrane support (Millipore, Bedford, MA, USA) were incubated with 5 μg/mL of anti-IFN-γ for 18 h at 4 °C. Next, the plates were washed and blocked with RPMI containing 10% FCS for 2 h at room temperature. Cells (5×10^5 cells/well) in RPMI with 1% FCS were distributed in duplicates in microplates in the presence of medium alone, or hamster monoclonal anti-mouse CD3 (1 μg/mL, Pharmingen), or 10 μg/mL of HIV-1 Gag epitope restricted to H-2^d MHC class I AMQMLKETINEEAAE$_{197–211,}$ or 10 μg/mL of HIV-1 Gag epitope restricted to H-2^d MHC class II-restricted VDRFYKTLRAEQASQ$_{297–308}$ epitope (NIH AIDS Research and Reference Reagent Program), or with 2 μg/mL of p24Gag from HIV-1 or with 10 μg/mL of 25 pools containing five HIV Gag peptides (15-mers of the HIV-1 HXB2gag peptide, with overlap of 11 residues, totaling 123 peptides and named according to the amino acid sequence of Gag), or for 18 h at 37 °C in 5% CO_2. After the washes, the plates were incubated with biotinylated anti-IFN-γ for 2 h at room temperature. The plates were washed and incubated with avidin-peroxidase and developed with 3-amino-9-ethylcarbazole substrate (Calbiochem). Spots were quantified with an Immunospot Imager Analyzer using the software ImmunoSpot 3.2 (CTL ImmunoSpot® S4 Analyzer, C.T.L., Cleveland, OH, USA). The reaction was considered positive when the number of spot-forming cells (SFCs) was equal to or higher than 10 SFC/10^6 cells. All results are expressed as the mean number of SFCs per 10^6 MSCs.

Cytokine measurement

Mononuclear spleen cells (MSC) were cultured in 96-well microplates (Costar, Cambridge, MA) with 10 μg/mL of the MHC class I-restricted AMQMLKETINEEAAE$_{197–211}$ epitope from HIV Gag protein (NIH AIDS

Research) and incubated at 37 °C in 5% CO_2. Supernatants were collected after 72 h and stored at −70 °C. Cytokine quantification was conducted using an Inflammation mouse kit (BD Pharmingen) and analyzed in a BD FACSCalibur cytometer (BD Biosciences, San Jose, CA USA).

Proliferation assay with [^{3}H]thymidine

MSCs were cultured in 96-well microplates (Costar) containing 5 μg/mL of the H-2^d MHC class I-restricted AMQMLKETINEEAAE$_{197–211}$ or the class II-restricted VDRFYKTLRAEQASQ$_{297–311}$ epitope from HIV Gag protein (NIH AIDS Research) or 2 μg of p24Gag at 37 °C in 5% CO_2. Thymidine incorporation was measured on day 6 of the culture, 18 h after being pulsed with 1 μCi [3H]thymidine (GE Healthcare, Little Chalfont, UK).

Proliferation assay with CFSE

MSCs were stained with 5 μM of CFSE (carboxyfluorescein-succinimidyl-ester, e-Bioscience, California, USA) at 37 °C for 30 min, washed twice with RPMI + 10% FCS, adjusted to 1×10^6 cells/mL and cultured with 1 μg of p24Gag or 5 μg of Gag class I epitope AMQMLKETINEEAAE$_{197–211}$ for six days at 37 °C in 5% CO_2. After six days, the cells were collected and stained with mouse anti-CD4 PC5 and mouse anti-CD8 PE and analyzed in a four-color flow cytometer (Epics XL, Beckman Coulter, CA, USA). A minimum of 100,000 events were analyzed, and the results are expressed as the percentage of CFSE low/CD4+ and CFSE low/CD8+ cells.

Evaluation of CD4+ or CD8+ T cell responses

After neonatal immunization, mice were sacrificed, and MSCs were collected at 35 days or at the end of the long-term evaluation (6 or 9 months old).

Pentamer staining

MSCs were incubated with PE-conjugated H-2K^d/AMQMLKETI HIV Gag (MHC Class I pentamers) and FITC-conjugated anti-CD8 (ProImmune, Bradenton, USA) for 30 min. After the washes, the cells were analyzed using four-color direct flow cytometry (Epics XL, Beckman Coulter, CA, USA). A minimum of 500,000 events were analyzed, and the results are expressed as the percentage of pentamer+/CD8+ cells.

Extracellular staining

MSCs were cultured for six hours with 5 μg of Gag MHC class I and II epitope to H-2^d. Cells were collected, washed and stained using the antibodies PerCP-conjugated rat anti-mouse-CD4 and anti-mouse-CD8, PE-conjugated anti-mouse-CD127 and FITC-conjugated anti-mouse-CD69 (all purchased from BD Pharmingen) for 30 min at 4 °C in the dark. After the washes, the cells were analyzed using four-color direct flow cytometry (Epics XL, Beckman Coulter, CA, USA). A minimum of 50,000 events were analyzed, and the results are expressed as the percentage of CD4+ or CD8+ expressing CD69.

Intracellular staining

To evaluate granzyme expression, MSCs were stained using extracellular markers (PerCP-conjugated anti-mouse-CD8 and FITC-conjugated anti-mouse-CD107a). To evaluate production of IFN-γ, TNF-α and IL-2, MSCs were incubated with class I or II Gag epitopes for six hours, and 10 μg/mL brefeldin A (Sigma) was added after 2 h of incubation at 37 °C. Next, 5×10^5 cells were stained with PC5-conjugated anti-mouse CD8 or anti-CD4 (BD Pharmingen) for 30 min. After the washes, the cells were fixed with 4% paraformaldehyde for 10 min at 4 °C and incubated with PBS, 0.5% saponin (Sigma) and the following antibodies: PE-conjugated rat anti-mouse-Granzyme B (e-Bioscience, San Diego, CA, USA), PE-conjugated rat anti-mouse-IFN-γ, PE-conjugated rat anti-mouse-IL-2, FITC-conjugated rat anti-mouse-TNF-α or Ig isotype control rat Ab (all purchased from BD Pharmingen) for 40 min at 4 °C. Fluorescence data for 50,000 events were obtained in a four-color direct flow cytometer (Epics XL, Beckman Coulter, CA, USA). The results are expressed as the percentage of CD4+ or CD8+ co-expressing IFN-γ/TNF-α or IFN-γ/IL-2 or only one of these cytokines.

In vivo analysis of CTL activity

To evaluate CTL activity *in vivo*, neonatal mice were immunized at 7 days old (do) and 25 do (5 μg), and ten days later, they received the stained target cells, a modified version of a previously described protocol (Valentin et al., 2009). MSCs from non-immunized BALB/c mice were used as target cells and were stained with high (10 μM) or low (1 μM) concentrations of carboxyfluorescein-succinimidyl-ester (CFSE, Molecular Probes, USA) in RPMI medium for 30 min at 37 °C in the dark. The population stained with high-concentration CFSE was pulsed with 2 μg of class I peptide AMQMLKETI$_{197–205}$ for one hour at 37 °C. Target cells with low and high concentrations of CFSE were washed and mixed at a 1:1 proportion in PBS solution. Approximately 3×10^7 cells in 200 μL of PBS were injected i.v. into immunized mice. Splenocytes of immunized mice were collected after 18 h, and transferred target cells were evaluated based on high and low intensities of CFSE by flow cytometry. The percentage of lysis was determined by the following calculation: % of specific lysis = 100 − {[(% of CFSE high cells IM / % of CFSE low cells IM) / (% of CFSE high cells NI / % of CFSE low cells NI)] × 100}. IM = immunized; NI = non-immunized.

Statistics

Values for all measurements are expressed as means ± SEM. Data from two groups were considered statistically significant when the *P* value was <0.05 using the *Mann–Whitney* test, whereas three or more groups were analyzed using the Kruskall–Wallis test with Dunn's post-test. The data were analyzed in GraphPad Prism 3.0 (GraphPad, San Diego, California, USA).

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