Virology **303,** 327–337 (2002)
doi:10.1006/viro.2002.1647

Expression of IL-18 by SIV Does Not Modify the Outcome of the Antiviral Immune Response

Luis D. Giavedoni,*,‡,[1] M. Cristina Velasquillo,* Laura M. Parodi,* Gene B. Hubbard,†,‡ and Vida L. Hodara*

*Department of Virology and Immunology, †Department of Laboratory Animal Medicine, and ‡Southwest Regional Primate Research Center, Southwest Foundation for Biomedical Research, San Antonio, Texas 78245-0549

Received December 14, 2001; returned to author for revision April 15, 2002; accepted June 5, 2002

Interleukin 18 (IL-18) is a proinflammatory cytokine expressed by several cell types, including activated dendritic cells and macrophages, that acts in synergy with IL-12 as an important amplifying factor for IFN-γ production and Th1 development. To study the immunological and virological effects of IL-18 expression in the context of a lentiviral infection, we inoculated rhesus macaques with a high dose of replication-competent simian immunodeficiency virus (SIV) vectors carrying the rhesus IL-18 gene in the sense (SIV_{IL-18}) or antisense (SIV_{FIGI}) orientation. Both vectors behaved as attenuated viruses, resulting in low viral loads, induction of low and transient levels of inflammatory cytokines, no $CD4^+$ T cell depletion, and mild activation of T lymphocytes. Although IL-18-expressing virus could be isolated from some SIV_{IL18}-infected macaques for 12 weeks postinfection, the anti-SIV humoral and cellular immune responses of macaques inoculated with SIV_{IL18} and SIV_{FIGI} were similar to each other, with the exception of an early IFN-γ response in animals infected with SIV_{IL18}. In summary, expression of IL-18 during the acute phase of SIV infection does not increase viral replication or influence the outcome of the antiviral immune response.

Key Words: SIV; rhesus macaques; IL-18; immune response; live-attenuated virus; vaccine; cytokines; CTL; antibodies; proliferation.

INTRODUCTION

Interleukin-18, or interferon-γ inducing factor (IGIF), is a proinflammatory cytokine that plays an important role in the T cell helper type 1 (Th1) immune response, primarily by its ability to induce interferon-γ (IFN-γ) production in T cells and natural killer (NK) cells (Okamura *et al.,* 1995; Ushio *et al.,* 1996). IL-18 is expressed by a number of cells, including keratinocytes, osteoblasts, pituitary gland cells, adrenal cortical cells, intestinal epithelial cells, and, more importantly, antigen-presenting cells such as dendritic cells and activated macrophages (Dinarello, 1999; Lebel-Binay *et al.,* 2000). On the basis of its primary structure, three-dimensional folding, receptor family, signal transduction pathways, and biological effects, IL-18 appears to be a new member of the IL-1 family (Dinarello, 1999).

In the past few years, a large body of experimental evidence has supported the notion of employing IL-18 as an immune modulator, in synergy with IL-12, to enhance the generation of cell-mediated immune responses. IL-18 has been delivered as a protein preparation or as plasmid DNA and has boosted immune responses against cancer cells (Hara *et al.,* 2000; Nagai *et al.,* 2000; Yamanaka *et al.,* 1999), pathogens (Kremer *et al.,* 1999; Ohkusu *et al.,* 2000), and subunit antigens (Eberl *et al.,* 2000). More importantly, delivery of plasmid DNA coding for IL-18 has been shown to increase cell-mediated immune responses in mice (Billaut-Mulota *et al.,* 2000; Sin *et al.,* 1999) and in nonhuman primates (Kim *et al.,* 1999). These results make IL-18 an attractive candidate as a cytokine with potential for enhancing the cellular immune response to live-attenuated SIV vaccines. However, some recent publications demonstrated that IL-18 increased HIV-1 viral replication in cell lines (Klein *et al.,* 2000; Shapiro *et al.,* 1998), suggesting that attenuation could be jeopardized by IL-18 in the context of retroviral replication. In this regard, we recently reported the cloning of the rhesus macaque IL-18 gene and the construction of SIV vectors carrying this gene in the sense (SIV_{IL18}) and antisense (SIV_{FIGI}) orientation. Although SIV_{IL18} expressed bioactive IL-18, we did not observe increased *in vitro* viral replication in rhesus peripheral blood mononuclear cell (PBMC) (Giavedoni *et al.,* 2001). In this article, we report that expression of IL-18 by SIV *in vivo* does not result in increased viral replication. On the contrary, SIV vectors containing the rhesus IL-18 gene are attenuated and result in mild infection, and the infected animals are able to mount very effective immune responses.

RESULTS

Viral stocks of SIV_{IL-18} and SIV_{FIGI} were prepared based on the results of the *in vitro* characterization of all the recombinant SIV vectors that we generated (Giavedoni *et*

[1] To whom correspondence and reprint requests should be addressed at Southwest Foundation for Biomedical Research, P.O. Box 760549, San Antonio, TX 78245-0549. Fax: 210-670-3310. E-mail: Lgiavedo@icarus.sfbr.org.

0042-6822/02 $35.00

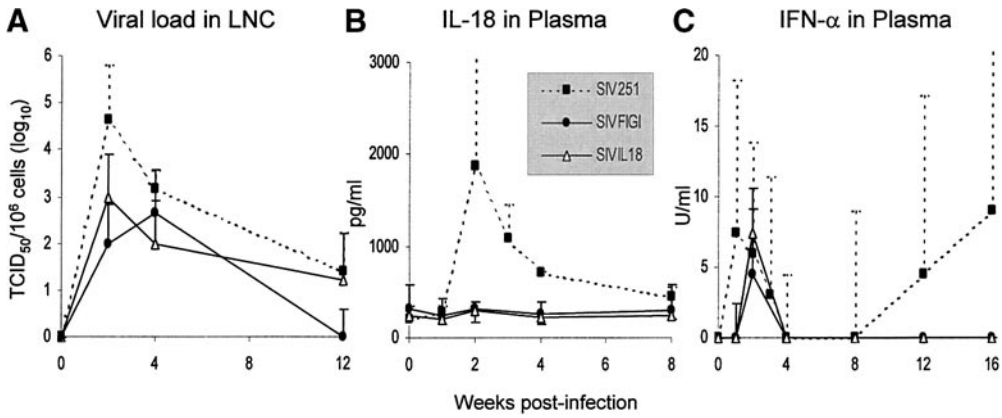

FIG. 1. Viral loads in lymph node cells and plasmatic cytokines in rhesus macaques after infection with SIV vectors. Rhesus macaques were infected with 10^4 $TCID_{50}$ of SIV_{FIGI} (closed circles) or SIV_{IL-18} (open triangles). (A) Cell-associated viral loads were determined in LNC by limited dilution and cocultivation with CEM-x-174 cells. (B) IL-18 concentration was measured by ELISA. (C) IFN-α-induced antiviral activity was detected in plasma by a biological assay as described under Materials and Methods. Values represent the mean of six animals, with standard deviation. As a comparison, similar measurements from four macaques infected with 10^2 $TCID_{50}$ of SIV_{mac251} are included (closed squares) (Giavedoni *et al.,* 2000).

al., 2001). SIV_{IL-18} contained the gene coding for the precursor form of the rhesus IL-18 protein in the sense orientation, whereas SIV_{FIGI} had the same nucleotide sequences in the antisense orientation. SIV_{IL-18} expressed bioactive IL-18 in rhesus PBMC, while SIV_{FIGI} served as a control that had similar genomic modifications and expressed no bioactive molecules. Twelve rhesus macaques were chosen by the ability of their PBMC to support SIV replication. Animals were divided into two groups of six monkeys each, with each group consisting of three male and three female monkeys of similar age and weight. Animals in one group were inoculated with 10^4 $TCID_{50}$ of SIV_{FIGI} iv, whereas macaques in the other group received the same dose of SIV_{IL18}.

Primary infection of rhesus macaques with SIV vectors

Macaques were intensively studied during the early phase of viral infection. Plasma samples were used for the determination of SIVp27, IL-4, IL-18, GM-CSF, IFN-α/β, and IFN-γ; lymph node (LN) biopsies were performed at 0, 2, 4, and 12 weeks postinfection (WPI). Data generated from this study were compared with our findings in rhesus macaques of similar age and genetic origin that were inoculated with a lower dose (100 $TCID_{50}$) of pathogenic SIV (Giavedoni *et al.,* 2000). Animals infected with recombinant SIV vectors had cell-associated viral loads in LN cells that were almost 100 times lower at 2 WPI than the ones observed in macaques infected with wild-type SIV; by 4 WPI, viral loads in these monkeys were similar to the ones observed at 2 WPI and comparable to the viral loads seen in wild-type infected monkeys at the same time point. Finally, by 12 WPI, viral loads reached a very low set-point (Fig. 1A), and virus isolation from the blood of macaques inoculated with the SIV vectors became sporadic from that time point forward (data not shown). Another striking difference with animals infected with pathogenic SIV was that macaques inoculated with SIV vectors did not show the classical peak of plasma IL-18 at 2 WPI, even for those animals infected with SIV_{IL18} (Fig. 1B). Macaques infected with SIV_{IL18} and SIV_{FIGI} also showed a transient peak of IFNα/β at 2 WPI, a delay of 1 week compared with macaques infected with SIV_{mac251} (Fig. 1C). Levels of SIVp27, GM-CSF, IL-4, and IFN-γ in plasma of macaques infected with SIV_{IL-18} and SIV_{FIGI} were below the limit of detection of the enzyme-linked immunosorbent assay (ELISA) tests at all time points.

Similarly, infection with SIV_{IL18} and SIV_{FIGI} did not result in the dramatic changes in the level of LN $CD4^+$ and $CD8^+$ T lymphocytes usually seen in macaques infected with SIV_{mac251} (Figs. 2A and 2B). Interestingly, macaques infected with SIV vectors did show the same pattern of NK cell activation seen in animals infected with pathogenic SIV (Fig. 2C). However, activation of $CD4^+$ and $CD8^+$ T cells in LN, measured as upregulation of CD69, was marginal (Figs. 3A and 3C). Histological evaluation of LNs during the same period showed mild classical changes associated with SIV infection with minimal to moderate hyperplasia that subsided by 12 WPI (data not shown). Activation of T lymphocytes in the peripheral blood of macaques infected with SIV vectors followed similar curves to the ones observed in animals infected with virulent SIV, although the percentage of activated cells was much lower (Figs. 3B and 3D). For example, upregulation of CD69 on T cells of macaques after infection (2 WPI) was seen for both monkeys infected with SIV_{IL18} (P values of 0.025 and 0.005 for $CD4^+$ and $CD8^+$ T cells, respectively) as well as monkeys infected with SIV_{FIGI} (P values are 0.048 and 0.0214 for $CD4^+$ and $CD8^+$ T cells, respectively).

In summary, primary infection of rhesus monkeys with

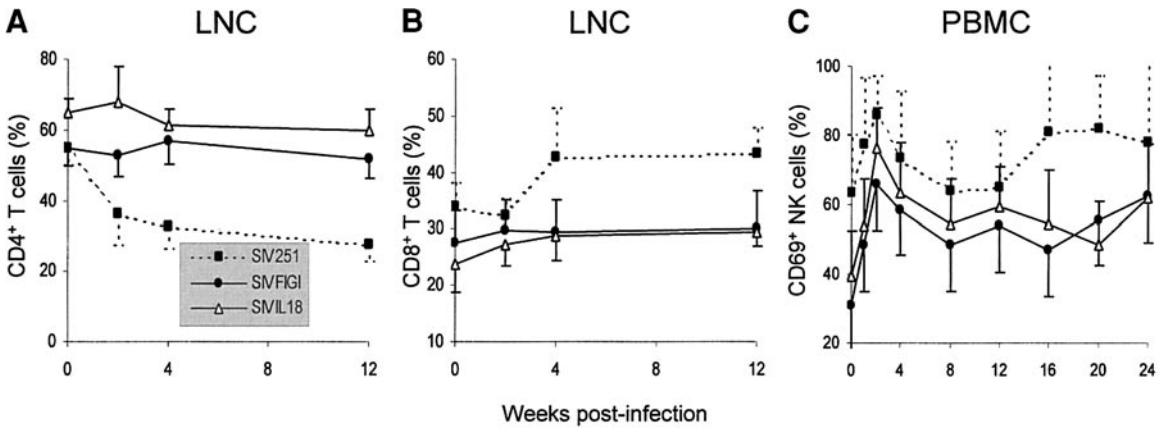

FIG. 2. Changes in lymphocyte phenotype after infection with SIV vectors. Rhesus macaques were infected with SIV_{FIGI} (closed circles) or SIV_{IL-18} (open triangles). Minimal changes were observed in $CD4^+$ and $CD8^+$ $CD3^+$ cell levels in lymph nodes (A and B, respectively), and for activation of NK cells ($CD3^-CD16^+CD56^+$) in peripheral blood (C). As a comparison, similar measurements from macaques infected with SIV_{mac251} are included (closed squares) (Giavedoni *et al.,* 2000). Cells were identified by flow cytometry as described under Materials and Methods. Values represent the mean with the standard deviation.

SIV_{IL18} was indistinguishable from infection with SIV_{FIGI} since both resulted in low viral loads and very mild cellular and inflammatory changes. Thus, expression of IL-18 by SIV *in vivo* does not increase viral loads or lymphocyte activation.

Viral species isolated from infected animals

Virus was isolated from the LN of rhesus macaques at different time points after infection and the 3′ LTR of the isolated proviral DNA was characterized by PCR with

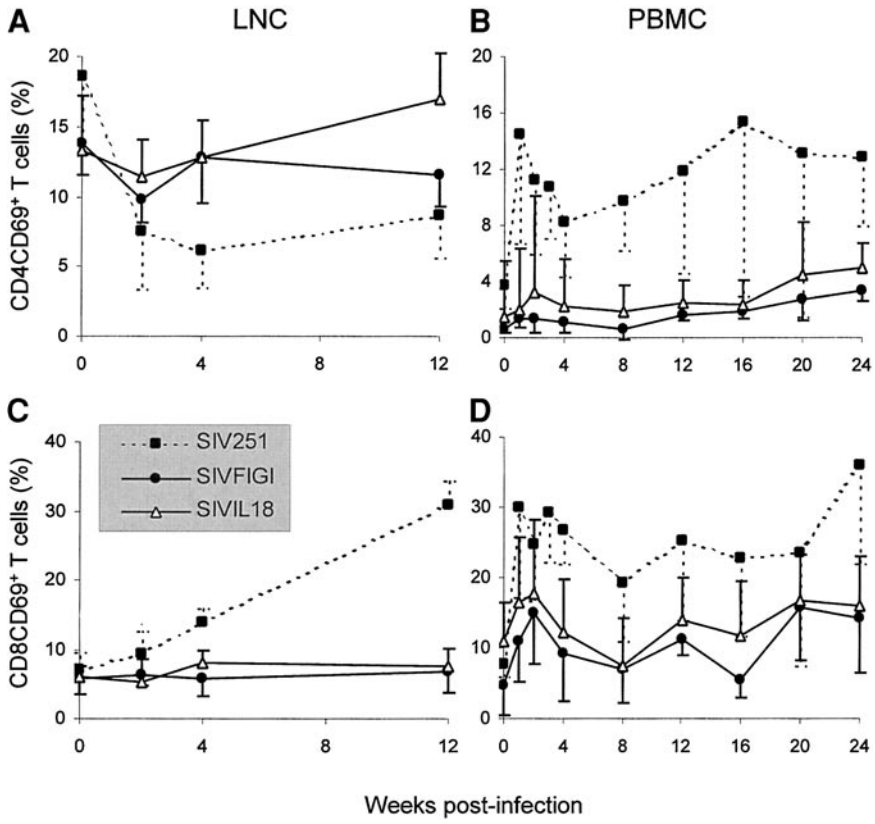

FIG. 3. Mild activation of $CD3^+$ T cells after infection with SIV vectors. Changes in the level of expression of the activation marker CD69 on $CD4^+$ (A and B) and $CD8^+$ (C and D) T cells present in lymph nodes (A and C) or peripheral blood (B and D) were determined by flow cytometry. Animals were infected with SIV_{FIGI} (closed circles) or SIV_{IL-18} (open triangles). Similar measurements from macaques infected with SIV_{mac251} are included (closed squares) (Giavedoni *et al.,* 2000).

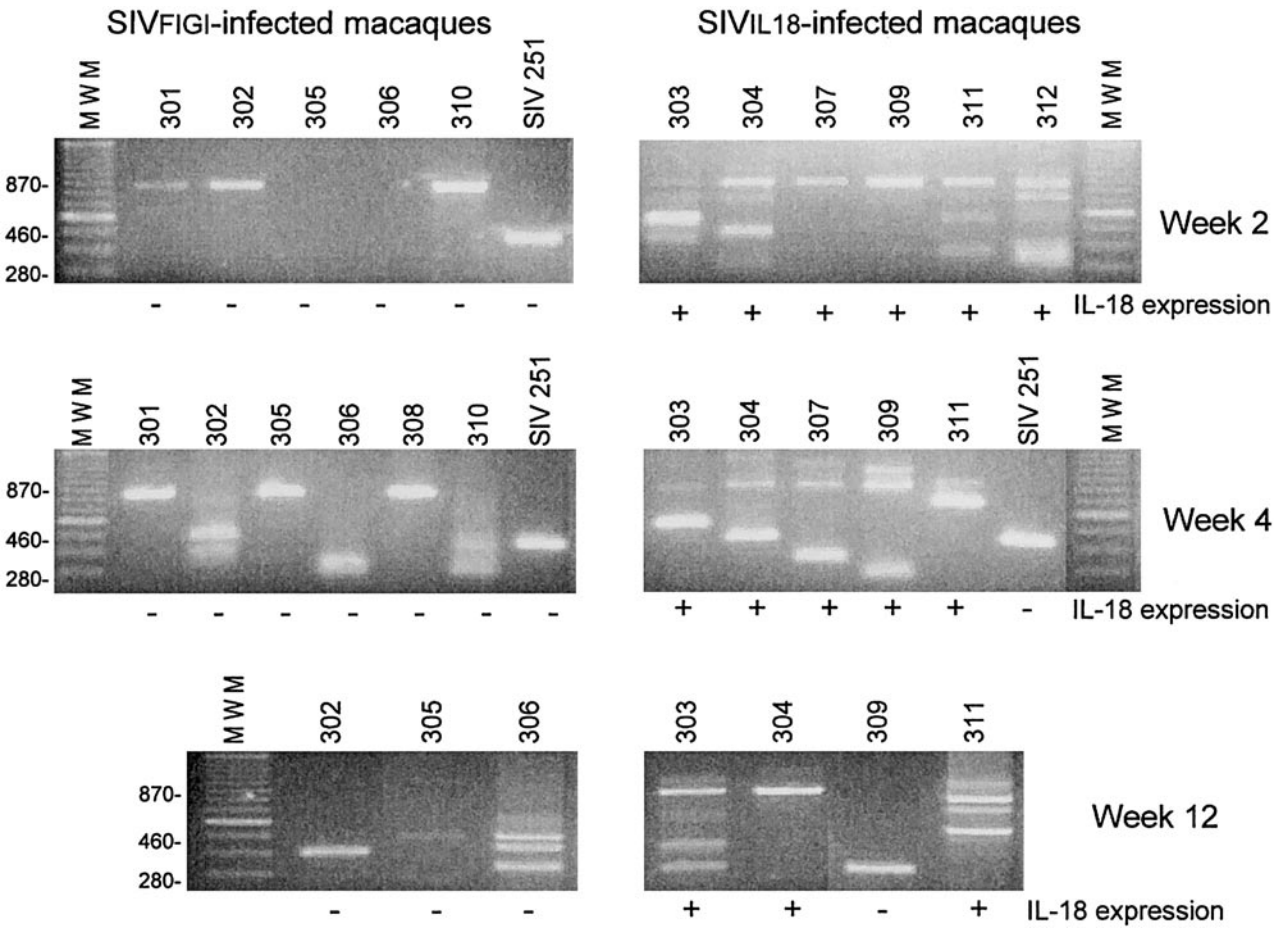

FIG. 4. Characterization of proviral DNA in lymph node cells from macaques infected with SIV_{IL18} or SIV_{FIGI}. Virus isolated by cocultivation of LN cells and CEM-x-174 cells was characterized by PCR as described under Materials and Methods. PCR products were separated in a 1% agarose gel. MWM: 100-bp ladder. The supernatant of each coculture was assayed for the presence of IL-18 by ELISA and the KG-1 biological assay as described under Materials and Methods.

primers that bound to SIV regions surrounding the site where the IL-18 gene had been inserted. The supernatant of these cocultures was also evaluated for the presence of IL-18 by an ELISA specific for the biologically active molecule (Shida *et al.,* 2001; Taniguchi *et al.,* 1997) and the KG-1 biological assay (Konishi *et al.,* 1997). By 2 WPI, viruses isolated from macaques infected with SIV_{IL18} and SIV_{FIGI} showed the presence of full-length IL-18 gene inserts, although the appearance of viral species in which the insert was being deleted was already evident (Fig. 4). By 4 WPI, the accumulation of viruses that were losing the IL-18 gene insert was more evident, even though bioactive IL-18 continued to be expressed. By 12 WPI, three of the four macaques inoculated with SIV_{IL18} from which virus could be isolated still expressed bioactive IL-18. Finally, by 16 WPI all the isolated viruses had lost the IL-18 gene insert (data not shown).

Thus, SIV vectors inevitably lose their IL-18 gene inserts and evolve into species that have an average "wild-type virus" size genomes. There was a tendency for a more rapid deletion of the inserted sequences in animals inoculated with SIV_{FIGI} than in macaques infected with SIV_{IL18}. This phenomenon has been observed before and it seems related to a particularly unstable arrange of nucleotides in the viral genome rather than a direct effect of the cytokine being expressed (Giavedoni *et al.,* 1997). At all time points, IL-18 expression and activity was found only in the supernatant of positive cultures from SIV_{IL18}-infected animals, indicating that PBMC or LN cells from which nonrecombinant SIV is isolated *in vitro* do not normally release IL-18.

Immune responses of rhesus macaques to SIV vectors

The cellular and humoral SIV-specific immune responses of the inoculated macaques were evaluated by different methodologies. Humoral immune responses were determined by antigen-specific ELISA (Fig. 5). Both anti-gp160 and anti-Gag antibodies were detected in some animals by 4 WPI and in all monkeys by 8 WPI and reached high and stable levels by 12 WPI. Differences in

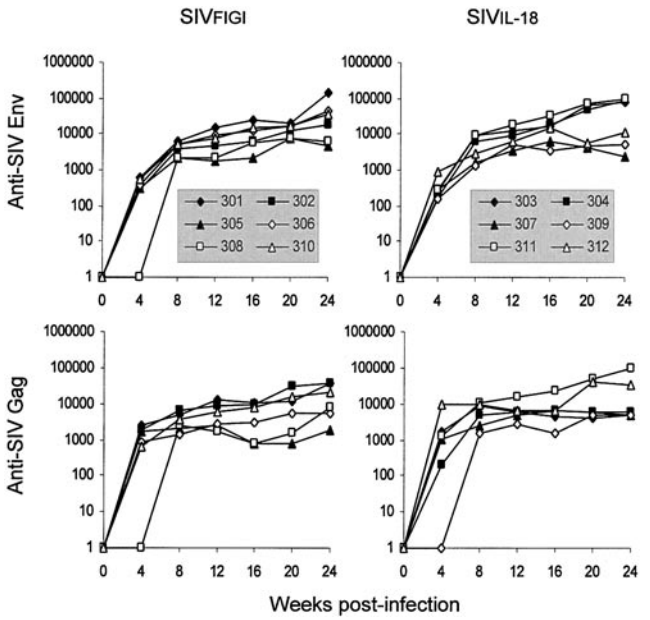

FIG. 5. Humoral immune responses of macaques inoculated with SIV vectors. Titer of antibodies specific for SIV Env (top row) or SIV Gag (bottom row) were determined by antigen-specific ELISA as described under Materials and Methods.

titers between animals infected with SIV_{FIGI} or SIV_{IL18} were not statistically significant, indicating that antibody responses were not affected by expression of IL-18.

Proliferative immune responses specific for SIV were determined at 24 and 40 WPI against SIV Gag virus-like particles produced in insect cells by a recombinant baculovirus, using VSV nucleocapsid as a control antigen (Fig. 6). A two-tailed, two-sample unequal variance *t* test analysis of the data obtained at 24 WPI showed that, after stimulation with SIV Gag, cells from macaques inoculated with SIV_{IL18} released more IFN-γ than cells from monkeys infected with SIV_{FIGI} ($P = 0.036$). The same type of analysis on samples obtained at 40 WPI showed that the SIV Gag-specific expression of IFN-γ was not statistically significant between the two groups of animals ($P = 0.493$). As a control, the same cells exposed to the superantigens *Staphylococcus* enterotoxin A and B showed similar levels of expression of IFN-γ at 24 WPI ($P = 0.534$) and 40 WPI ($P = 0.630$).

We developed a nonradioactive killing assay using overlapping peptides to identify SIV Gag-specific CTL epitopes in animals infected with SIV_{IL18} and SIV_{FIGI} (Fig.

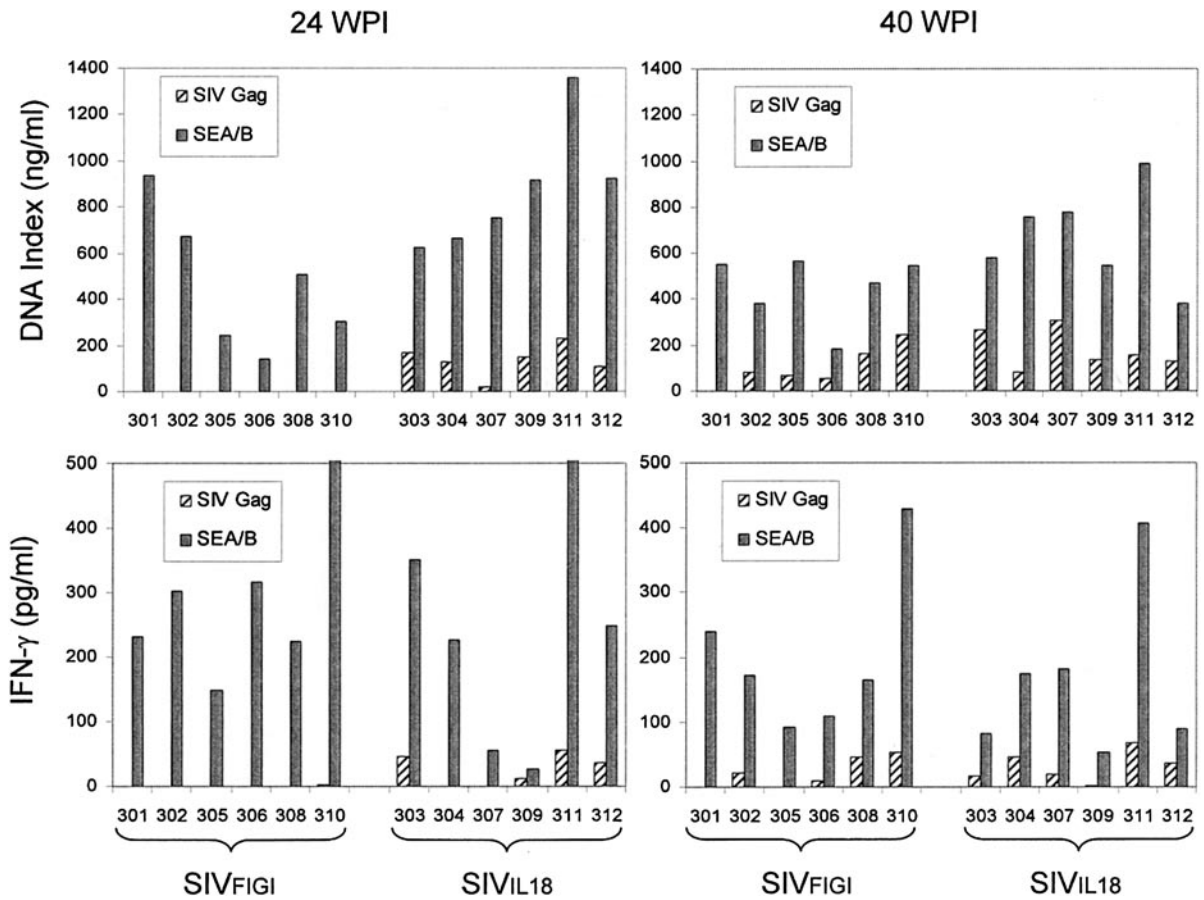

FIG. 6. Proliferation of macaque PBMC. PBMC from macaques inoculated with SIV_{IL18} or SIV_{FIGI} were harvested at 24 or 40 weeks postinoculation and exposed to either SIV Gag, vesicular stomatitis virus nucleoprotein (VSV-N), or the superantigens SEA and SEB. After 6 days in culture, IFN-γ was quantitated in the supernatant by ELISA and the cellular content was determined by DNA concentration. The value for wells containing VSV-N was subtracted from the values for SIV Gag and SEA/B.

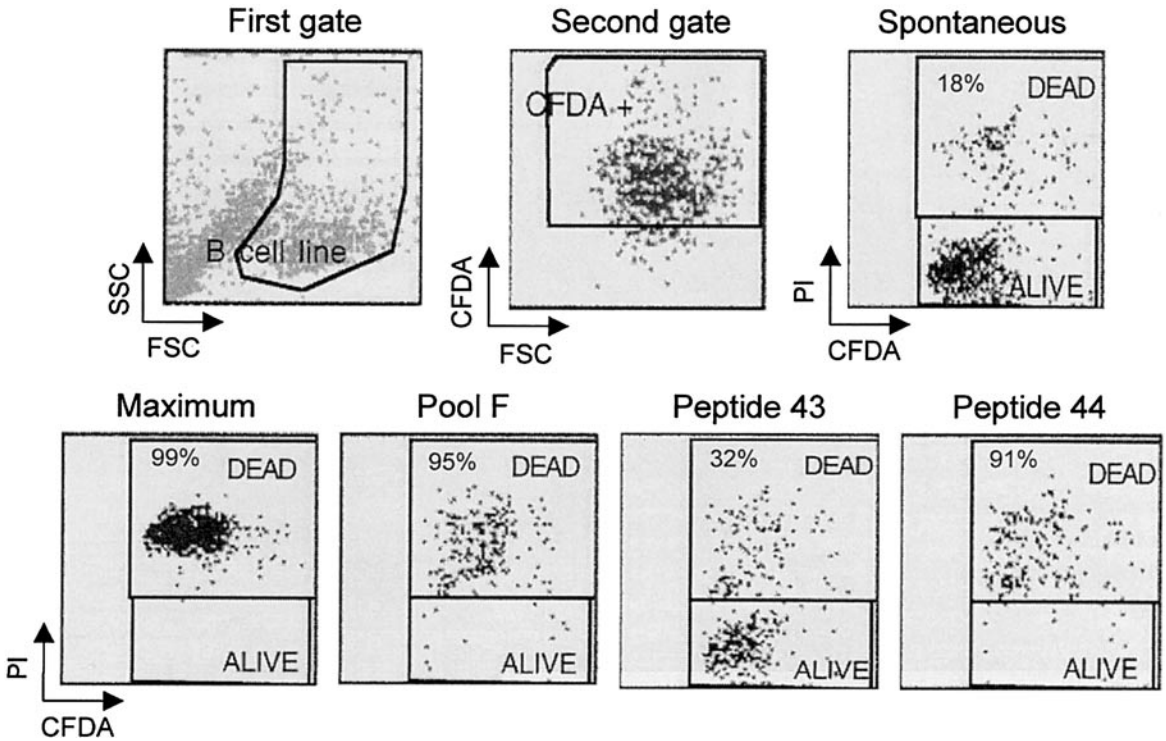

FIG. 7. Nonradioactive cell killing assay. B-LCL cells were stained with CFDA, incubated with SIV Gag peptides (pools of eight peptides or individual peptides), and exposed to stimulated autologous monkey PBMC. After a 4-h incubation, cells were stained with propidium iodide (PI), washed, and run in a flow cytometer. A first gate was created around the B-LCL. A second gate selected for CFDA-stained B-LCL. Finally, gated cells were analyzed in CFDA versus PI dot-plots. Spontaneous killing was determined in the absence of effector cells. Maximum killing consisted of target cells treated with saponin. The example shows PBMC from macaque 14305 killing autologous B-LCL that had been incubated with either SIV Gag peptide pool F (peptides 43 to 50, NIH AIDS Research and Reference Reagent Program), peptide 43 (DHVMAKCPDRQAGFLGLGPW), or reactive peptide 44 (QAGFLGLGPWGKKPRNFPMA).

7). This technique allowed us to identify CTL epitopes in 9 of the 12 macaques infected with the recombinant viruses. These reactive peptides were then used in IFN-γ ELISPOT assays to monitor the progression of the cellular immune response during SIV infection. Spot-forming cells (SFC) were found in animals from both groups, with notable intragroup animal-to-animal variation, and remained stable during the first 10 months of infection. Similarly, number of spots also varied for the same animal during time, with no correlation with viral loads. No statistically significant differences were observed when comparing the number of IFN-γ SFC from animals inoculated with SIV_{IL18} or SIV_{FIGI} (data not shown).

Hence, with the exception of an early IFN-γ immune response found in SIV_{IL18}-infected monkeys, anti-SIV humoral and cellular immune responses were similar for macaques infected with either SIV_{IL18} or SIV_{FIGI}.

DISCUSSION

The current perception in the field of AIDS vaccine development is that a candidate immunogen will have to elicit both strong and long-lasting CTLs and broadly neutralizing antibodies (Nabel, 2001). Live-attenuated viruses are the type of vaccines that have provided the best examples of protection in the SIV/macaque model (Daniel *et al.,* 1992; Giavedoni *et al.,* 1997; Joag *et al.,* 1998; Johnson *et al.,* 1999; Silverstein *et al.,* 2000; Villinger *et al.,* 2000). However, reports of residual pathogenicity (Baba *et al.,* 1999; Wyand *et al.,* 1997) and potential for recombination of the vaccine virus with challenge virus (Gundlach *et al.,* 2000) have reduced the original enthusiasm and made the prospect of such vaccine for humans almost impractical. Nevertheless, the study of host–pathogen interactions between rhesus macaques and attenuated SIV offers an excellent opportunity for identifying immune correlates for protection that could be applied to other vaccines. In general, live-attenuated vaccines have shown a direct correlation between virulence and immunogenicity (Desrosiers *et al.,* 1998; Johnson and Desrosiers, 1998). Therefore, we decided to explore ways to increase immunogenicity without compromising attenuation. Cytokines have been shown to serve as natural adjuvants in several experimental settings in nonhuman primates, for example, increasing the immunogenicity of DNA vaccines or changing the type of the induced immune response (Barouch *et al.,* 2000; Kim *et al.,* 1999). We have previously employed SIV as an expression vector for cytokines and showed

that the expression of IFN-γ by SIV resulted in an attenuated virus that induced low and transient viral loads in rhesus macaques (Giavedoni *et al.,* 1997). However, that study compared the infectivity of the SIV vector expressing IFN-γ (SIV_{HyIFN}) with the parental virus SIVΔnef, and those two viruses had different genome lengths and ability to replicate in rhesus PBMC (Giavedoni and Yilma, 1996). The possibility that the *in vivo* attenuation observed for the SIV vector expressing IFN-γ was due to its intrinsic difficulty for replication, rather than the biological activity of the cytokine being expressed, was not evaluated. In the present study we compared two viruses with identical genome length which differed only in the orientation of the inserted rhesus IL-18 gene and, therefore, in their capacity to express bioactive IL-18. Thus, differences in the virological and immunological outcomes of infection of rhesus macaques with SIV_{IL18} or SIV_{FIGI} would reflect the consequences of precursor IL-18 protein expression by SIV. Our results here show that both SIV_{IL18} and SIV_{FIGI} replicated at low levels in the infected animals, similarly to the slow *in vitro* replication observed in rhesus PBMC (Giavedoni *et al.,* 2001). Virus isolated from macaques inoculated with SIV_{IL18} during the peak of viremia still expressed bioactive IL-18, demonstrating that expression of the proinflammatory cytokine IL-18 *in vivo* did not result in increased SIV replication. However, due to this limited viral replication, we did not observe an increase in plasma levels of IL-18 in animals inoculated with SIV_{IL18} compared with the concentration observed in macaques inoculated with SIV_{FIGI}. In fact, as another indication of limited viral spread and replication, animals inoculated with both SIV vectors had undetectable levels of SIVp27 in plasma.

In a recent study, we reported that infection of rhesus macaques with pathogenic SIV_{mac251} induced the accumulation of IFN-α/β and IL-18 in plasma and that NK activity paralleled changes in viral loads (Giavedoni *et al.,* 2000). We now describe that macaques of similar genetic background, sex, and age, inoculated with a larger dose of SIV vectors carrying the IL-18 gene, have a very different plasma cytokine profile. For example, the peak of IFN-α/β, although still present, was detectable only at 2 WPI, and there was no increment in the concentration of IL-18 during this acute phase. However, activation of NK cells was very similar to the one seen in monkeys infected wild pathogenic virus. Since both IFN-α and IL-18 are cytokines released by dendritic cells and stimulated macrophages that induce activation of NK cells during viral infections (Matikainen *et al.,* 2001; Sareneva *et al.,* 1998), our findings indicate that IFN-α may be a more relevant mediator of the innate immune response during SIV infection. Additionally, we demonstrate that macaques inoculated with SIV_{IL18} or SIV_{FIGI} show no statistically significant changes in the level of CD69 expression on $CD4^+$ or $CD8^+$ T cells in LNs and that the transient activation of $CD8^+$ T cells in peripheral blood may reflect the expansion of CTL that usually controls the primary SIV infection (Kuroda *et al.,* 1999). All these findings add to the notion that these SIV vectors are very attenuated and that expression of IL-18 has a limited role on SIV replication.

The influence of IL-18 expression by SIV on the generation of an anti-SIV immune response was very modest. IL-18 has been shown to act synergistically with IL-12 in generating Th1 immune responses to DNA-encoded antigens (Billaut-Mulota *et al.,* 2000; Kremer *et al.,* 1999; Sin *et al.,* 1999) and proteins (Eberl *et al.,* 2000). However, it was recently demonstrated in mice that aberrant expression of IL-18 resulted in increased production of both Th1 and Th2 cytokines (Chan *et al.,* 2001) and that IL-18 induced the differentiation of Th1 or Th2 cells depending upon cytokine milieu and the genetic background of the animals (Xu *et al.,* 2000). These findings have led to the understanding that the functions of IL-18 *in vivo* are very heterogeneous and complicated; in principle, IL-18 enhances the IL-12-driven Th1 immune responses, but it can also stimulate Th2 immune responses in the absence of IL-12 (Nakanishi *et al.,* 2001). In our experimental model, although IL-18-expressing virus was rescued from three of six animals inoculated with SIV_{IL18} for over 3 months, their humoral or cellular immune responses were not that different from those detected in the other SIV_{IL18}- or SIV_{FIGI}-infected macaques. The only distinguishable and measurable effect of IL-18 expression by SIV on the immune response was an early *in vitro* IFN-γ response to SIV Gag in animals inoculated with SIV_{IL18}. Then again, despite this IFN-γ response, anti-SIV Gag antibodies and CTL activities measured at identical time points were similar for animals inoculated with SIV_{IL18} or SIV_{FIGI}.

It is important to mention that both SIV_{IL18} or SIV_{FIGI} lack *nef* coding sequences. The *nef* gene product of SIV has several biological features, such as downregulation of the CD3 ζ-chain, CD4, CD28, and class I MHC expression, and association with many intracellular proteins (reviewed in Geyer *et al.,* 2001) that may affect directly or indirectly the generation of an immune response. Therefore, another possibility to explain our results is that infection with slow replicating viruses that lack *nef* expression allows the macaque's immune system to mount an efficient response, which is not affected by the low expression level of IL-18.

In summary, we demonstrated that recombinant SIV vectors that carry the IL-18 gene in either direction behave similar to live-attenuated viruses *in vivo,* resulting in low viral loads, efficient antiviral immune response, and lack of generalized cell activation. Expression of IL-18, a proinflammatory cytokine known to increase HIV replication *in vitro,* did not result in increased SIV replication or in any other detectable change in virological and immunological parameters. Perhaps the expression by SIV of other cytokines with a more potent and direct effect on

the immune system, such as IL-12, may have a more dramatic effect in enhancing the immune response to live-attenuated SIV vaccines.

MATERIALS AND METHODS

Cells and viruses

The construction and characterization of the replication-competent SIV vector expressing rhesus IL-18 has been described elsewhere (Giavedoni *et al.,* 2001). Briefly, SIV_{IL18} and SIV_{FIGI} are SIV_{mac239} viruses with a deletion in the *nef* gene and with an insertion of the rhesus macaque IL-18 gene that codes for the precursor protein in the sense and antisense orientation, respectively. CEM-x-174 cells, rhesus PBMCs, and lymph node cells were used for SIV isolation and propagation. These cells, and the NK-sensitive human erythroblastoid cell line K562, were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, 0.1 mg/ml streptomycin, and 100 U/ml penicillin (Cellgro Mediatech, Herndon, VA) [RPMI-10]. Human A549 cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum and antibiotics. Encephalomyocarditis virus (EMCV), used for the antiviral assay of IFN in plasma, was propagated in human A549 cells.

Infection of rhesus macaques

Twelve colony-bred, weight-, sex-, and age-matched rhesus macaques (*Macaca mulatta*) were used in this experiment. Animals were seronegative for simian type D retroviruses, simian T cell leukemia virus, and SIV. The animals were used and cared for in accordance with the American Association for Accreditation of Laboratory Animal Care Guidelines, and protocols were approved by the SFBR Institutional Animal Care and Use Committee (IACUC). Six macaques (14303, 14304, 14307, 14309, 14311, and 14312) were inoculated iv with 1 ml of RPMI 1640 containing 10^4 $TCID_{50}$ of SIV_{IL18}. The remaining animals (14301, 14302, 14305, 14306, 14308, and 14310) received 10^4 $TCID_{50}$ of the control virus SIV_{FIGI}. Each group consisted of three male and three female monkeys.

Measurements in plasma

Plasma p27 antigenemia was measured by a commercial SIV core antigen capture ELISA (Coulter Corp., Hialeah, FL) as instructed by the manufacturer (sensitivity of 50 pg/ml). Levels of GM-CSF, IL-4, IL-12, and IFN-γ were determined with commercially available ELISA kits (kits for GM-CSF and IL-4 were from Immunotech, Miami, FL, and Cytoscreen Monkey IFN-γ and IL-12 were from BioSource, Camarillo, CA). The limits of detection were 5 pg/ml for GM-CSF and IL-4, and 4 pg/ml for IFN-γ and IL-12. Plasma levels of IL-18 were determined with an ELISA kit kindly provided by H. Okamura (Hyogo College of Medicine and Hayashibara Corp., Japan), as described before (Taniguchi *et al.,* 1997); the monoclonal antibodies of this ELISA kit only recognize bioactive IL-18 with a sensitivity of 10 pg/ml (Shida *et al.,* 2001). Plasma IFN activity was determined by measuring inhibition of the cytopathic effect caused by EMCV infection in A549 cells (Giavedoni *et al.,* 1992). IFN-α was also determined in plasma with a commercial kit (PBL Biomedical Laboratories, New Brunswick, NJ).

LN biopsies

Peripheral LN (axillary and/or inguinal) were obtained by transcutaneous biopsy under ketamine HCl anesthesia (10 mg/kg im; Parke-Davis, Morris Plains, NJ) prior to and at 2, 4, and 12 WPI. LN were divided aseptically into two fractions for single-cell preparation and pathological analysis. Lymphocyte suspensions were obtained by mechanical teasing of tissues. The fractions for pathological analysis were fixed in 10% neutral buffered formalin, processed conventionally, cut at 5 μm, and stained with hematoxylin and eosin (H&E) for routine histological examination. Tissues were examined blinded for changes in lymphoid architecture and cellularity.

Cell-associated viral loads

Cell-associated virus, latent or productive, was measured by limiting-dilution assay of LN cells cocultured with CEM-x-174 cells in 24-well plates (Van Rompay *et al.,* 1992). Twice weekly, culture media were assayed for the presence of the SIV major core protein (p27) by ELISA (Lohman *et al.,* 1991). Virus levels were calculated according to the method of Reed and Muench (1938) and expressed as $TCID_{50}$ per 10^6 cells. Cell-free supernatants were tested for IL-18 biological activity on KG-1 cells as described before (Giavedoni *et al.,* 2001).

Isolation of proviral DNA

Proviral DNA was extracted from 1×10^6 LNC, PBMC, or CEM-x-174 cells and amplified by PCR with primers specific for the SIV 3′ region. Primers for PCR amplification were H (sense primer, $^{5'}$CTCTCTGCGACCCTACAGAGG$^{3'}$, nt 9272 of SIV) and E ($^{5'}$AAATCCCTTCCAGTCCCCCC$^{3'}$, antisense, nt 9710 of SIV). Nucleic acids were denatured at 94°C for 1 min, annealed at 65°C for 1 min, and extended at 72°C for 2 min; this cycle was repeated 35 times. PCR products were resolved in 1% agarose gels. DNA fragments generated by this reaction were 870 nt in length for viruses containing the intact IL-18 gene insert, 460 nt for wild-type SIV, and 280 nt for $SIV_{\Delta nef}$, respectively.

Lymphocyte phenotyping

Phenotypic characterization of PBMC and LNC was performed by flow cytometry using three-color direct immunofluorescence as described before (Giavedoni *et al.,* 2000). Absolute values for cells in whole blood were obtained by combining the percentages obtained by flow cytometry with the values of total white blood cell count per microliter and the differential formula for each animal at each time point.

Anti-SIV antibodies

Plasma samples were analyzed for the presence of antibodies reactive to SIV envelope glycoproteins and p27 core protein. Antigens for ELISA plates were obtained from a viral preparation of SIVmac239 concentrated by 20% sucrose cushion centrifugation. Protein content of the viral preparation was determined with the CBQCA Protein Quantification Kit (Molecular Probes, Eugene, OR). Anti-gp160 antibodies were quantitated with lysed SIV particles and concavalin A coated plates as previously described (Cole *et al.,* 1997; Robinson *et al.,* 1990). For the anti-SIV p27 antibody ELISA, the same disrupted, envelope-depleted viral preparation was added to ELISA plates previously coated with anti-SIV p27 antibodies (Lohman *et al.,* 1991).

Proliferation assay

Proliferative responses of rhesus lymphocytes to different antigens were tested *in vitro.* Rhesus PBMC were isolated and seeded into a round-bottom polypropylene 96-well plate at a density of 1×10^5 in 200 μl RPMI 10 medium. PBMC were stimulated for 6 days in the presence of SIVgag VLPs (3 μg/ml), vesicular stomatitis virus nucleoprotein (VSV N) (3 μg/ml), or *Staphylococcus* enterotoxin A and B (SEA/B) (0.5 μg/ml). On day 6, supernatant (150 μl) was harvested to test for cytokine production by an in-house IFN-γ ELISA. Volume was restored by adding back 150 μl RPMI and then an aliquot (20 μl) of the cell suspension was removed to determine cell density by a fluorescent DNA quantitation assay (CyQUANT cell proliferation assay, Molecular Probes). The IFN-γ ELISA plates were prepared by coating Immulon II plates with 5 μg/ml of a murine mAb anti-human IFN-γ (clone MD-1, Biosource) in PBS. ELISA plates were coated for 3 h at 37°C, washed four times with 0.05% Tween-20-PBS, and blocked with blotto overnight at 4°C. Plates were washed again and 100 μl of supernatant and standards were loaded and incubated for 2 h at 37°C. Plates were washed and a 1:1000 dilution of a biotinylated rabbit polyclonal anti-IFN-γ antibody (BioSource, 100 μl/well) was added and incubated for 1 h at 37°C. After washing, plates were incubated with streptavidin-horseradish peroxidase (Bio-Rad, Richmond, CA) for 30 min at room temperature. After a final wash, plates were developed with 100 μl of TM-Blue substrate and stopped with 100 μl 2 N H_2SO_4. The OD of each was measured at 450 nm in an automated plate reader (HTS 7000 Bio Assay Reader, Perkin–Elmer, Branchburg, NJ).

Cytotoxic T lymphocyte assays

Autologous lymphoblastoid B cell lines (B-LCL) were prepared at the initiation of this study for each animal by infection of rhesus PBMC with herpes papio virus in the presence of cyclosporin A. A set of 48 peptides that span the whole SIVmac239 Gag protein, each 20 amino acids long and with a 10 amino acid overlap between sequential peptides (obtained from the NIH AIDS Research and Reference Reagent Program), were divided into six pools of eight continuous peptides. Rhesus PBMC were resuspended at 8×10^6 cells/ml in RPMI-10 and added in aliquots of 250 μl to six wells of a 48-well plate. Peptide pools were added to the cells at 10 μg/ml and cultured at 37°C for 48 h. On the third day, recombinant human IL-2 (a generous gift of Hoffmann-La Roche, Inc.) was added at 50 U/ml, and cells were kept in culture for a total of 14 days with the addition of fresh IL-2-containing medium twice per week. B-LCL (2×10^6 cells) were then treated with 50 μg/ml mitomycin C for 30 min at 37°C, washed thoroughly, divided in six aliquots of 2×10^5 each, and mixed with the different peptide pools for 90 min at 37°C. These B-LCL/peptide mixtures were used to stimulate autologous PBMC for an additional 3-day period. Finally, stimulated PBMC were purified by Ficoll–Hypaque centrifugation, counted, and tested in a flow cytometry killing assay, using B cell lines coated with SIV Gag peptide pools as the target. The day before the killing assay, B-LCL were washed with plain RPMI, resuspended at 5×10^7 cells/ml, and stained with 0.5 μM of 5- (and 6-)carboxy-fluorescein diacetate succinimidyl ester (CFDA, Molecular Probes) at 37°C for 10 min. The reaction was stopped with cold RPMI 10 and the B-LCL were incubated overnight at 37°C. The following morning, CFDA-labeled B-LCL were counted, divided into seven aliquots, and incubated with the different SIV Gag peptide pools for 90 min at 37°C. Stimulated PBMC were counted, added to CFDA-labeled B-LCL at different effector/target ratios, and incubated at 37°C for 4 h. At the end of the incubation, propidium iodine (PI) was added to each tube at 1 μg/ml, incubated at 37°C for 15 min, washed with 0.1% BSA in PBS, and analyzed by flow cytometry. The acquisition was performed with a first gate created around the B-LCL in a forward versus side-scatter dot-plot. A second gate was created for CFDA-positive cells in a forward-scatter versus FL-1 dot-plot. Finally, cells were analyzed in a FL-1 (FITC) versus FL-3 (PI) dot-plot, using B-LCL that had not been exposed to PBMC or that were treated with saponin to draw the spontaneous or maximum killing regions, respectively.

ELISPOT

Rhesus PBMC were seeded at 1–2 × 10^5 cells in 100 μl of RPMI 10 in IFN-γ ELISPOT plates (U-Cytech, Utrecht, The Netherlands), and reactive or control SIV Gag peptides were added in duplicate at 10 μg/ml. After a 24-h incubation at 37°C, cells were lysed and plates were developed according to the manufacturer's instructions. Spots were counted and the difference between the number of spots for the reactive peptide minus the number of spots for the peptide control was multiplied by 10 and referred to as spot-forming cells (SFC)/10^6 PBMC.

Statistical analyses

The two-sample *t* test for samples with unequal variances was implemented in the commercially available computer program SYSTAT, v.9.0 (SPSS, Inc., Chicago, IL).

ACKNOWLEDGMENTS

This work was funded by NIH Grants RO1 AI41923, R21 AI44363, and R51 RR13986. The authors thank Dr. Kathy Brasky, Robert Geiger, and personnel from the Department of Laboratory of Animal Medicine of the Southwest Foundation for assistance with the animal studies. We also thank M. Silva for technical assistance, Dr. Jon Allan for critical reading and comments of this manuscript, and A. Hopstetter for editing this document.

REFERENCES

Baba, T. W., Liska, V., Khimani, A. H., Ray, N. B., Dailey, P. J., Penninck, D., Bronson, R., Greene, M. F., McClure, H. M., Martin, L. N., and Ruprecht, R. M. (1999). Live attenuated, multiply deleted simian immunodeficiency virus causes AIDS in infant and adult macaques [see comments] [published erratum appears in *Nat. Med.* **5**(5), 590 (1999)]. *Nat. Med.* **5**(2), 194–203.

Barouch, D. H., Santra, S., Schmitz, J. E., Kuroda, M. J., Fu, T. M., Wagner, W., Bilska, M., Craiu, A., Zheng, X. X., Krivulka, G. R., Beaudry, K., Lifton, M. A., Nickerson, C. E., Trigona, W. L., Punt, K., Freed, D. C., Guan, L., Dubey, S., Casimiro, D., Simon, A., Davies, M. E., Chastain, M., Strom, T. B., Gelman, R. S., Montefiori, D. C., and Lewis, M. G. (2000). Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. *Science* **290**(5491), 486–492.

Billaut-Mulota, O., Idzioreka, T., Bana, E., Kremerb, L., Duprea, L., Loyensa, M., Riveaua, G., Lochtb, C., Caprona, A., and Bahra, G. M. (2000). Interleukin-18 modulates immune responses induced by HIV-1 Nef DNA prime/protein boost vaccine. *Vaccine* **19**(1), 95–102.

Chan, W. L., Pejnovic, N., Lee, C. A., and Al-Ali, N. A. (2001). Human il-18 receptor and st2l are stable and selective markers for the respective type 1 and type 2 circulating lymphocytes. *J. Immunol.* **167**(3), 1238–1244.

Cole, K. S., Rowles, J. L., Jagerski, B. A., Murphey-Corb, M., Unangst, T., Clements, J. E., Robinson, J., Wyand, M. S., Desrosiers, R. C., and Montelaro, R. C. (1997). Evolution of envelope-specific antibody responses in monkeys experimentally infected or immunized with simian immunodeficiency virus and its association with the development of protective immunity. *J. Virol.* **71**(7), 5069–5079.

Daniel, M. D., Kirchhoff, F., Czajak, S. C., Sehgal, P. K., and Desrosiers, R. C. (1992). Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. *Science* **258**(5090), 1938–1941.

Desrosiers, R. C., Lifson, J. D., Gibbs, J. S., Czajak, S. C., Howe, A. Y., Arthur, L. O., and Johnson, R. P. (1998). Identification of highly attenuated mutants of simian immunodeficiency virus. *J. Virol.* **72**(2), 1431–1437.

Dinarello, C. A. (1999). Interleukin-18. *Methods* **19**(1), 121–132.

Eberl, M., Beck, E., Coulson, C. P., Okamura, H., Wilson, W. R., and Mountford, M. A. (2000). IL-18 potentiates the adjuvant properties of IL-12 in the induction of a strong Th1 type immune response against a recombinant antigen. *Vaccine* **18**(19), 2002–2008.

Geyer, M., Fackler, O. T., and Peterlin, B. M. (2001). Structure-function relationships in HIV-1 Nef. *EMBO Rep.* **2**(7), 580–585.

Giavedoni, L., Ahmad, S., Jones, L., and Yilma, T. (1997). Expression of gamma interferon by simian immunodeficiency virus increases attenuation and reduces postchallenge virus load in vaccinated rhesus macaques. *J. Virol.* **71**(2), 866–872.

Giavedoni, L. D., Imhoof, J. D., Velasquillo, M. C., Parodi, L. M., and Hodara, V. L. (2001). Expression of the interleukin-18 gene from rhesus macaque by the simian immunodeficiency virus does not result in increased viral replication. *J. Interferon Cytokine Res.* **21,** 173–180.

Giavedoni, L. D., Jones, L., Gardner, M. B., Gibson, H. L., Ng, C. T., Barr, P. J., and Yilma, T. (1992). Vaccinia virus recombinants expressing chimeric proteins of human immunodeficiency virus and gamma interferon are attenuated for nude mice. *Proc. Natl. Acad. Sci. USA* **89**(8), 3409–3413.

Giavedoni, L. D., Velasquillo, M. C., Parodi, L. M., Hubbard, G. B., and Hodara, V. L. (2000). Cytokine expression, natural killer cell activation, and phenotypic changes in lymphoid cells from rhesus macaques during acute infection with pathogenic simian immunodeficiency virus. *J. Virol.* **74**(4), 1648–1657.

Giavedoni, L. D., and Yilma, T. (1996). Construction and characterization of replication-competent simian immunodeficiency virus vectors that express gamma interferon. *J. Virol.* **70**(4), 2247–2251.

Gundlach, B. R., Lewis, M. G., Sopper, S., Schnell, T., Sodroski, J., Stahl-Hennig, C., and Uberla, K. (2000). Evidence for recombination of live, attenuated immunodeficiency virus vaccine with challenge virus to a more virulent strain. *J. Virol.* **74**(8), 3537–3542.

Hara, I., Nagai, H., Miyake, H., Yamanaka, K., Hara, S., Micallef, M. J., Kurimoto, M., Gohji, K., Arakawa, S., Ichihashi, M., and Kamidono, S. (2000). Effectiveness of cancer vaccine therapy using cells transduced with the interleukin-12 gene combined with systemic interleukin-18 administration. *Cancer Gene Ther.* **7**(1), 83–90.

Joag, S. V., Liu, Z. Q., Stephens, E. B., Smith, M. S., Kumar, A., Li, Z., Wang, C., Sheffer, D., Jia, F., Foresman, L., Adany, I., Lifson, J., McClure, H. M., and Narayan, O. (1998). Oral immunization of macaques with attenuated vaccine virus induces protection against vaginally transmitted AIDS. *J. Virol.* **72**(11), 9069–9078.

Johnson, R. P., and Desrosiers, R. C. (1998). Protective immunity induced by live attenuated simian immunodeficiency virus. *Curr. Opin. Immunol.* **10**(4), 436–443.

Johnson, R. P., Lifson, J. D., Czajak, S. C., Cole, K. S., Manson, K. H., Glickman, R., Yang, J., Montefiori, D. C., Montelaro, R., Wyand, M. S., and Desrosiers, R. C. (1999). Highly attenuated vaccine strains of simian immunodeficiency virus protect against vaginal challenge: inverse relationship of degree of protection with level of attenuation. *J. Virol.* **73**(6), 4952–4961.

Kim, J. J., Nottingham, L. K., Tsai, A., Lee, D. J., Maguire, H. C., Oh, J., Dentchev, T., Manson, K. H., Wyand, M. S., Agadjanyan, M. G., Ugen, K. E., and Weiner, D. B. (1999). Antigen-specific humoral and cellular immune responses can be modulated in rhesus macaques through the use of IFN-gamma, IL-12, or IL-18 gene adjuvants. *J. Med. Primatol.* **28**(4–5), 214–223.

Klein, S. A., Klebba, C., Kauschat, D., Pape, M., Ozmen, L., Hoelzer, D., Ottmann, O. G., and Kalina, U. (2000). Interleukin-18 stimulates HIV-1 replication in a T-cell line. *Eur. Cytokine Netw.* **11**(1), 47–52.

Konishi, K., Tanabe, F., Taniguchi, M., Yamauchi, H., Tanimoto, T., Ikeda, M., Orita, K., and Kurimoto, M. (1997). A simple and sensitive bioassay for the detection of human interleukin-18/interferon-gamma-in-

ducing factor using human myelomonocytic KG-1 cells. *J. Immunol. Methods* **209**(2), 187–191.

Kremer, L., Dupre, L., Wolowczuk, I., and Locht, C. (1999). *In vivo* immunomodulation following intradermal injection with DNA encoding IL-18. *J. Immunol.* **163**(6), 3226–3231.

Kuroda, M. J., Schmitz, J. E., Charini, W. A., Nickerson, C. E., Lifton, M. A., Lord, C. I., Forman, M. A., and Letvin, N. L. (1999). Emergence of CTL coincides with clearance of virus during primary simian immunodeficiency virus infection in rhesus monkeys. *J. Immunol.* **162**(9), 5127–5133.

Lebel-Binay, S., Berger, A., Zinzindohoue, F., Cugnenc, P., Thiounn, N., Fridman, W. H., and Pages, F. (2000). Interleukin-18: Biological properties and clinical implications. *Eur. Cytokine Netw.* **11**(1), 15–26.

Lohman, B. L., Higgins, J., Marthas, M. L., Marx, P. A., and Pedersen, N. C. (1991). Development of simian immunodeficiency virus isolation, titration, and neutralization assays which use whole blood from rhesus monkeys and an antigen capture enzyme-linked immunosorbent assay. *J. Clin. Microbiol.* **29**(10), 2187–2192.

Matikainen, S., Paananen, A., Miettinen, M., Kurimoto, M., Timonen, T., Julkunen, I., and Sareneva, T. (2001). IFN-alpha and IL-18 synergistically enhance IFN-gamma production in human NK cells: Differential regulation of Stat4 activation and IFN-gamma gene expression by IFN-alpha and IL-12. *Eur. J. Immunol.* **31**(7), 2236–2245.

Nabel, G. J. (2001). Challenges and opportunities for development of an AIDS vaccine. *Nature* **410**(6831), 1002–1007.

Nagai, H., Hara, I., Horikawa, T., Fujii, M., Kurimoto, M., Kamidono, S., and Ichihashi, M. (2000). Antitumor effects on mouse melanoma elicited by local secretion of interleukin-12 and their enhancement by treatment with interleukin-18. *Cancer Invest.* **18**(3), 206–213.

Nakanishi, K., Yoshimoto, T., Tsutsui, H., and Okamura, H. (2001). Interleukin-18 regulates both Th1 and Th2 responses. *Annu. Rev. Immunol.* **19,** 423–474.

Ohkusu, K., Yoshimoto, T., Takeda, K., Ogura, T., Kashiwamura, S., Iwakura, Y., Akira, S., Okamura, H., and Nakanishi, K. (2000). Potentiality of interleukin-18 as a useful reagent for treatment and prevention of Leishmania major infection. *Infect. Immunol.* **68**(5), 2449–2456.

Okamura, H., Tsutsi, H., Komatsu, T., Yutsudo, M., Hakura, A., Tanimoto, T., Torigoe, K., Okura, T., Nukada, Y., Hattori, K., and *et al.* (1995). Cloning of a new cytokine that induces IFN-gamma production by T cells [see comments]. *Nature* **378**(6552), 88–91.

Reed, L. J., and Müench, H. (1938). A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* **27,** 493–497.

Robinson, J. E., Holton, D., Liu, J., McMurdo, H., Murciano, A., and Gohd, R. (1990). A novel enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to HIV-1 envelope glycoproteins based on immobilization of viral glycoproteins in microtiter wells coated with concanavalin A. *J. Immunol. Methods* **132**(1), 63–71.

Sareneva, T., Matikainen, S., Kurimoto, M., and Julkunen, I. (1998). Influenza A virus-induced IFN-alpha/beta and IL-18 synergistically enhance IFN-gamma gene expression in human T cells. *J. Immunol.* **160**(12), 6032–6038.

Shapiro, L., Puren, A. J., Barton, H. A., Novick, D., Peskind, R. L., Shenkar, R., Gu, Y., Su, M. S., and Dinarello, C. A. (1998). Interleukin 18 stimulates HIV type 1 in monocytic cells. *Proc. Natl. Acad. Sci. USA* **95**(21), 12550–12555.

Shida, K., Shiratori, I., Matsumoto, M., Fukumori, Y., Matsuhisa, A., Kikkawa, S., Tsuji, S., Okamura, H., Toyoshima, K., and Seya, T. (2001). An alternative form of IL-18 in human blood plasma: Complex formation with IgM defined by monoclonal antibodies. *J. Immunol.* **166**(11), 6671–6679.

Silverstein, P. S., Mackay, G. A., Mukherjee, S., Li, Z., Piatak, M., Jr., Lifson, J. D., Narayan, O., and Kumar, A. (2000). Pathogenic simian/human immunodeficiency virus SHIV(KU) inoculated into immunized macaques caused infection, but virus burdens progressively declined with time. *J. Virol.* **74**(22), 10489–10497.

Sin, J. I., Kim, J. J., Boyer, J. D., Ciccarelli, R. B., Higgins, T. J., and Weiner, D. B. (1999). *In vivo* modulation of vaccine-induced immune responses toward a Th1 phenotype increases potency and vaccine effectiveness in a herpes simplex virus type 2 mouse model. *J. Virol.* **73**(1), 501–509.

Taniguchi, M., Nagaoka, K., Kunikata, T., Kayano, T., Yamauchi, H., Nakamura, S., Ikeda, M., Orita, K., and Kurimoto, M. (1997). Characterization of anti-human interleukin-18 (IL-18)/interferon-gamma-inducing factor (IGIF) monoclonal antibodies and their application in the measurement of human IL-18 by ELISA. *J. Immunol. Methods* **206**(1–2), 107–113.

Ushio, S., Namba, M., Okura, T., Hattori, K., Nukada, Y., Akita, K., Tanabe, F., Konishi, K., Micallef, M., Fujii, M., Torigoe, K., Tanimoto, T., Fukuda, S., Ikeda, M., Okamura, H., and Kurimoto, M. (1996). Cloning of the cDNA for human IFN-gamma-inducing factor, expression in Escherichia coli, and studies on the biologic activities of the protein. *J. Immunol.* **156**(11), 4274–4279.

Van Rompay, K. K., Marthas, M. L., Ramos, R. A., Mandell, C. P., McGowan, E. K., Joye, S. M., and Pedersen, N. C. (1992). Simian immunodeficiency virus (SIV) infection of infant rhesus macaques as a model to test antiretroviral drug prophylaxis and therapy: Oral 3′-azido-3′-deoxythymidine prevents SIV infection. *Antimicrob. Agents Chemother.* **36**(11), 2381–2386.

Villinger, F., Switzer, W. M., Parekh, B. S., Otten, R. A., Adams, D., Shanmugam, V., Bostik, P., Mayne, A. E., Chikkala, N. F., McClure, H. M., Novembre, F., Yao, Q., Heneine, W., Folks, T. M., and Ansari, A. A. (2000). Induction of long-term protective effects against heterologous challenge in SIVhu-infected macaques. *Virology* **278**(1), 194–206.

Wyand, M. S., Manson, K. H., Lackner, A. A., and Desrosiers, R. C. (1997). Resistance of neonatal monkeys to live attenuated vaccine strains of simian immunodeficiency virus. *Nat. Med.* **3**(1), 32–36.

Xu, D., Trajkovic, V., Hunter, D., Leung, B. P., Schulz, K., Gracie, J. A., McInnes, I. B., and Liew, F. Y. (2000). IL-18 induces the differentiation of Th1 or Th2 cells depending upon cytokine milieu and genetic background. *Eur. J. Immunol.* **30**(11), 3147–3156.

Yamanaka, K., Hara, I., Nagai, H., Miyake, H., Gohji, K., Micallef, M. J., Kurimoto, M., Arakawa, S., and Kamidono, S. (1999). Synergistic antitumor effects of interleukin-12 gene transfer and systemic administration of interleukin-18 in a mouse bladder cancer model. *Cancer Immunol. Immunother.* **48**(6), 297–302.