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Research paper

Use of retroviral vectors for the analysis of SIV/HIV-specific CD8 T cell responses

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Abstract

CD8$^+$ T cell responses and particularly cytotoxic T lymphocyte (CTL) activity are critical factors in controlling SHIV, SIV or HIV replication during natural infection and represent key parameters which need to be monitored during vaccine development. In order to improve the methodology for measuring CD8$^+$ T cell responses, retroviral vectors expressing the full-length SIV-Gag or HIV-Env proteins were constructed and used to transduce B lymphoblastoid cell lines (BLCL) from cynomolgus monkeys infected with SHIV89.6P. Continuous expression of Gag and Env proteins was detected in stably transduced BLCL, which induced Gag- or Env-specific T cell responses, as measured by both IFNγ – ELISPOT and chromium release assays, upon in vitro stimulation of PBMC from the SHIV89.6P-infected monkeys. Moreover, induction of Gag-specific CTL using BLCL transduced with retroviral vector expressing the SIV-Gag protein was more efficient and specific compared to that obtained using BLCL infected with a recombinant vaccinia virus (rVV) encoding for the same antigen. Assays on purified CD4$^+$ and CD8$^+$ T cells indicated that both populations specifically produced IFNγ, but only the CD8$^+$ T cells mediated Gag- and Env-specific cytotoxicity, indicating preferential expansion of these effector cells. Thus, this method represents an alternative tool for the analysis of CTL responses during vaccination protocols in those animal models where little information is available on MHC class I alleles or CTL epitopes.
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Keywords: CTL; Retroviral vector; SHIV/SIV; Vaccine; AIDS

1. Introduction

Accumulating evidence suggests that CD8$^+$ T cell responses and particularly cytotoxic T lymphocyte (CTL) activity play a critical role in controlling SHIV/SIV replication in infected macaques (Schmitz et al., 1999; Barouch et al., 2000) as well as HIV in humans (Ogg et al., 1998). For these reasons, they represent a key correlate of protection or nonprogression both in the natural infection and after vaccination. Recently, the quantification of antigen-specific CD8$^+$ T cell responses has become easier with the develop-

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ment of tetrameric MHC-peptide complexes (Kuroda et al., 1998; Donahoe et al., 2000). However, this assay relies predominantly on the identification of optimal epitopes and on the knowledge of MHC class I alleles, whereas, to date, information for MHC alleles of certain monkey species such as *Macaca fascicularis* (cynomolgus monkeys), an animal model of AIDS, is not available. In addition, since MHC tetramer binding only requires proper TCR expression, this assay does not provide any evidence on the functional capacity of labeled T cells. Other techniques are widely used for the monitoring of specific T cell responses, such as assays for the determination of cytokine production after antigen-specific stimulation (ELISPOT and intracellular staining). However, the significance of any relationship between the quantification of antigen-specific $CD8^+$ T cell responses measured by these latest approaches and the activity of antigen-specific $CD8^+$ CTL measured by the standard chromium release assay remains to be determined. Therefore, the opportunity to measure functionally the ability of $CD8^+$ T cells to lyse target cells remains an important tool. In this respect, recombinant vaccinia viruses (rVV) are widely used to deliver viral antigens to stimulator/target cells in chromium release and in IFNγ-ELISPOT assays (Rose et al., 2001; Sun et al., 2003).

Here, we report on the development and use of autologous B lymphoblastoid cell line (BLCL) stably transduced with retroviral vectors expressing the full-length SIV-Gag and HIV-Env proteins to measure CTL activity in cynomolgus monkeys infected with SHIV89.6P. Furthermore, we show that the induction of antigen-specific $CD8^+$ cytolytic T cells using retrovirus transduced BLCL is more efficient than that obtained using BLCL infected with rVV encoding for the same antigen. This technology allows the establishment of permanent cell lines constitutively expressing the target antigens and overcomes the safety concerns inherent with the handling of a replication competent rVV.

2. Methods

2.1. Animals

Two male cynomolgus macaques (*M. fascicularis*), Mk 760 and Mk 783, from the Mauritius breeding colony were housed in single cages within level three biosafety facilities in accordance with European guidelines for nonhuman primate care (EEC Directive no. 86-609, Nov. 24, 1986). For challenge, monkeys were infected intravenously with 20 MID_{50} of the SHIV89.6P virus. Clinical observations, weight measurements and blood sampling for haematological analysis and immunological assays were taken while under ketamine hydrochloride anaesthesia (10 mg/kg, i.m.). Volumes of 10 ml of whole blood were collected into an acid citrate-containing tube.

2.2. Production of retroviral vectors

A schematic diagram of the retroviral vectors used in this study is illustrated in Fig. 1. To express the SIV-Gag protein (SIVmac239 $p55_{Gag}$), two different retroviral vectors, pBabe-SIVGag.CTE and pFB-SIV-Gag.CTE, were produced by cloning the full-length SIV-Gag coding sequence linked to a cytoplasmic transporter element (CTE) for Rev/RRE independent nuclear export (Zolotukhin et al., 1994) into the *Sal*I site of the retroviral vectors pBabe-Puro (Morgenstern and Land, 1990) and pFB-Neo (Stratagene, La Jolla, CA, USA), respectively.

A retroviral vector expressing the HIV-1_{IIIB} envelope (Env) was obtained by cloning the gp160 full-length Env gene into the pBAF-Neo retroviral vector. The pBAF-Neo vector is a chimeric retroviral vector composed of a mixture of pBabe-Puro and pFB-Neo sequences. For pBAF-Neo construction, a 2.6 kb *Sac*I/*Sal*I DNA fragment from pFB-Neo was replaced with the corresponding 2.0 kb *Sac*I/*Sal*I DNA fragment from pBabe-Puro. The resulting retroviral cassette contains the 5′ elements from pBabe-Puro vector and the 3′ elements from the pFB-Neo vector. In this way, the pBAF-Neo vector lacks the splice acceptor and donor signals of the parental pFB-Neo plasmid, but retains the IRES-Neo-expressing cassette (Fig. 1). For the construction of the HIV-1_{IIIB} envelope-expressing retroviral vector pBAF-IIIBEnv, a 3.0 kb *Sal*I/*Eco*RI DNA fragment from pSVIIIenv3-2 (a gift from J. Sodroski), containing the full-length HXB2/IIIB envelope sequence and the full-length two-exon coding sequence of Rev, was cloned into the *Sal*I/*Eco*RI restriction sites of pBAF-Neo.

The resulting retroviral vectors pBabe-SIV-Gag.CTE (Babe-Gag), pFB-SIVGag.CTE (FB-Gag),

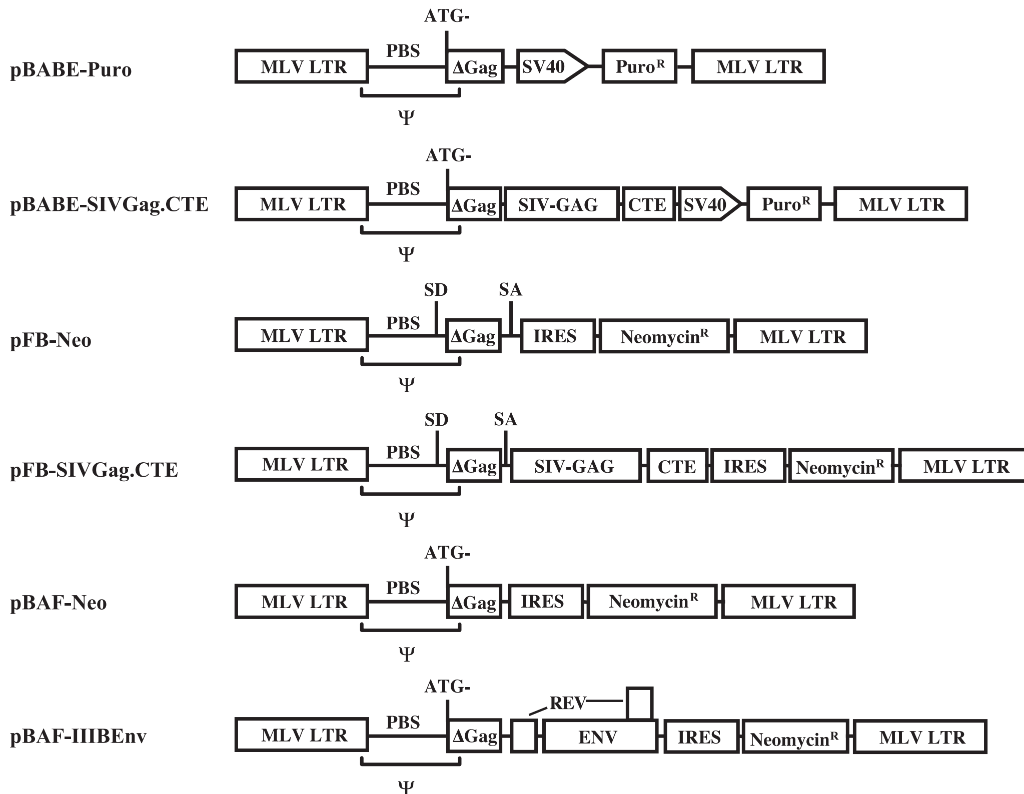

Fig. 1. Schematic representation of the retroviral vectors expressing SIV-Gag and HIV-Env used for BLCL transduction after the production of VSV-G pseudotyped recombinant retroviral vectors by transient transfection, as described in the Methods section. The packaging signal (ψ), primer binding site (PBS), the mutated Gag start codon (ATG-), splice donor (SD) and acceptor (SA) sites are indicated.

pBAF-IIIBEnv (BAF-Env), and the control vectors alone (Babe-Puro, FB-Neo and BAF-Neo) were transfected by the Calcium Phosphate method (Promega, Madison, WI, USA) into the Phoenix packaging cell line along with a VSV-G-expressing plasmid used to increase viral tropism and titer (Grignani et al., 1998). The retroviral vector containing supernatants were then analyzed for reverse transcriptase activity using standard methodologies (Weiss et al., 1982) and used for BLCL transduction.

2.3. Transduction of BLCL

In order to produce stimulator/target cells expressing the SIV-Gag or HIV-Env proteins, *Herpesvirus papio*-transformed B lymphoblastoid cell lines (BLCL) derived from cynomolgus monkeys were infected for 2 h at 37 °C in 5% CO_2 with Babe-Gag, FB-Gag-, BAF-Env-, Babe-Puro-, FB-Neo- and BAF-Neo-containing supernatants. After infection, cells were washed and then seeded in a 24-well plate at 0.5×10^6 cells/ml in RPMI 1640 (GIBCO Invitrogen, Paisley, UK) supplemented with L-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 μg/ml) and 15% heat-inactivated fetal bovine serum. After 48 h of culture, transduced BLCL (Retro-BLCL) were selected using either 5 μg/ml of puromycin (Sigma, St. Louis, MO, USA) for those transduced with Babe-Gag and Babe-Puro or 400 μg/ml of G418 (GIBCO) for those transduced with FB-Gag, BAF-Env, FB-Neo and BAF-Neo, and they were always kept under selection during the experiments. Production of SIV-Gag in cell lysates from Retro-BLCL stably transduced with Babe-Gag (Babe-Gag BLCL), FB-Gag (FB-Gag BLCL) or the control vectors (Babe-CTR BLCL and FB-CTR BLCL) was evaluated using an antigen capture ELISA procedure with a limit of detection of 20 pg/ml (Innotest, Innogenetics, Ghent,

Belgium). Production of HIV-Env in cell lysates from BLCL stably transduced with BAF-Env (BAF-Env BLCL) or the control vector (BAF-CTR BLCL) was evaluated using the HIV-1 gp120 antigen capture ELISA kit with a limit of detection of 310 pg/ml (Advanced Biotechnologies, Columbia, MD, USA).

2.4. ^{51}Chromium release assay

Peripheral blood mononuclear cells (PBMC) isolated from the animals were cultured in 24-well plates. Standard chromium release assays were performed using Retro-BLCL or recombinant vaccinia virus (rVV)-infected BLCL as stimulator and target cells. Concerning rVV infection, BLCL were infected overnight at 37 °C in 5% CO_2 with rVV containing either the full-length open reading frame of SIV $p55_{gag}$ (rVV-SIVGag) derived from the sequence of SIVmac239 (Delchambre et al., 1989) or the β-Galactosidase coding sequence (rVV-βGal) at a multiplicity of infection (moi) of 5 PFU/cell. Retro-BLCL- and rVV-infected BLCL (rVV-BLCL) were added on day 0 after irradiation (3000 rad) at an effector:target (*E*:*T*) ratio of 20:1. Human recombinant interleukin 2 (rIL-2) (5 U/ml) was added on day 8 and on every other 2 days. On day 14, chromium release assays and IFNγ-ELISPOT tests were performed. In the case of the chromium release assay, 5×10^3 target cells (Retro-BLCL and rVV-BLCL) were seeded in duplicate wells for each *E*:*T* ratio (from 50:1 to 2.5:1) after labeling with 50 μCi of ^{51}Cr (Perkin-Elmer Life Sciences, Boston, MA, USA) per 10^6 cells for 1 h. Chromium release was then assayed after 4 h of incubation at 37 °C in 5% CO_2 by harvesting 40 μl of supernatant from each well into the wells of a LumaPlate-96 (Perkin-Elmer Life Sciences). Emitted radioactivity was measured by a MicroBeta counter (Wallac, Oy Turky, Finland). Spontaneous release and total release were determined from wells containing target cells and medium alone or wells with added 100 μl of 0.1% Triton X-100 (Sigma). Percent specific cytotoxicity was calculated as [(test release − spontaneous release)/(total release − spontaneous release)] × 100. Spontaneous release of target cells was < 15% in all assays. The intra assay variability was determined on duplicate wells. The percent coefficient of variation (%CV) was always below 6.

2.5. IFNγ-ELISPOT assay

The IFNγ-ELISPOT assay was performed using reagents from Mabtech (Mabtech, Gamla Värmdöv, Sweden). Briefly, PBMC (effector cells, $0.5-1 \times 10^5$) isolated from infected monkeys and stimulated for 14 days as described above were resuspended in 100 μl and seeded in a microtiter plate (MultiScreen-IP plate, Millipore, Bedford, MA, USA) coated with a monoclonal antibody against monkey IFNγ. Stimulator cells (Retro-BLCL or rVV-BLCL) were resuspended in 100 μl of medium and added in duplicate to the plate at an *E*:*T* ratio of 20:1. After overnight incubation at 37 °C, cells were removed, a biotinylated antibody against monkey IFNγ was added to the wells, followed by the addition of a Streptavidin-alkaline phosphatase (ALP) and then of a chromogenic substrate (BCIP/NBT, Sigma). After development, spot-forming cells (SFC) were analyzed and counted using an ELISPOT reader (AID, Amplimedical Bioline, Turin, Italy).

2.6. Separation of effector cells

^{51}Cr release and IFNγ-ELISPOT assays were performed using total PBMC (bulk population), CD4-depleted or $CD4^+$ T cells as effector cells. For depletion, PBMC stimulated for 14 days with antigen-specific expressing BLCL were depleted of $CD4^+$ cells using MACS CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), following the manufacturer's instructions. The efficiency of the magnetic separation was evaluated by flow cytometry (FACScan, Becton Dickinson, San Josè, CA, USA) using FITC-conjugated anti-monkey CD3 (BioSource, Nivelles, Belgium), PE-conjugated anti-human CD4- and PercP-conjugated anti-human CD8 (Becton Dickinson) monoclonal antibodies.

3. Results

3.1. Expression of SHIV antigens by transduced BLCL

The expression of Gag and Env proteins was tested in transduced BLCL several times and in particular at the time each experiment was performed, using ELISA procedures, as described in the Methods section. SIV-Gag protein was constitutively produced at levels

Table 1
Production of SIV-Gag (p27) and HIV-Env (gp120) proteins in transduced BLCL and rVV-infected BLCL

BLCL	$p27_{gag}$ pg/10^6 cells (range[a])	$gp120_{env}$ pg/10^6 cells (range[a])
Babe-CTR	< 20	< 310
Babe-Gag	102–453	NT
FB-CTR	< 20	< 310
FB-Gag	380–1045	NT
BAF-CTR	< 20	< 310
BAF-ENV	NT	1054–1755
rVV-Gag	1402–1674	NT
rVV-βGal	< 20	NT

NT: Not tested.

[a] The expression of Gag and Env proteins was tested in stably transduced BLCL several times and in particular at the time each experiment was performed, using ELISA procedures as described in the Methods section.

ranging from 102 to 453 pg/10^6 cells in Babe-Gag BLCL and from 380 to 1045 pg/10^6 in FB-Gag BLCL, respectively (Table 1). rVV-Gag-infected BLCL showed high but transient expression of p27 (1402–1674 pg/10^6) after overnight incubation with rVV-Gag. Regarding the expression of Env protein, BAF-Env BLCL expressed level of gp120/10^6 cells ranging from 1054 to 1755 pg. Gag and Env proteins were not detectable in BLCL transduced with control retroviral vectors and in rVV-βGal BLCL (Table 1). Notably, transduced BLCL stably expressed the antigen of interest, without silencing the transgene throughout the entire period of the study (up to 2 years).

The transduced BLCL-expressing Gag and Env were then utilized as both stimulator and target cells for the analysis of antigen-specific CD8 responses in the SHIV89.6P-infected monkeys.

3.2. Induction of antigen-specific T cells

Firstly, Babe-Gag BLCL from Mk 760 infected with the SHIV89.6P were produced and used to evaluate the presence of antigen-specific T cells. The

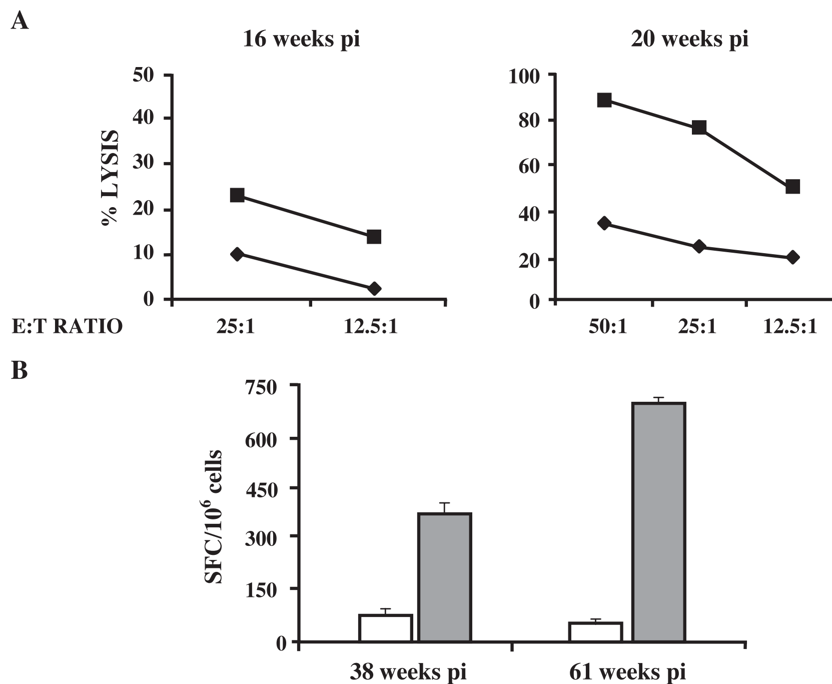

Fig. 2. (A) Anti-SIV Gag-specific CTL activity measured at different time points postinfection (pi) in Mk 760, infected with SHIV89.6P. At 16 and 20 weeks postinfection, PBMC from the infected monkey were stimulated for 14 days in the presence of autologous Babe-Gag BLCL. The percentage of specific lysis was determined by a standard ^{51}Cr release assay using the Babe-Gag BLCL (■) or the Babe-CTR BLCL (◆) as target cells. (B) SIV Gag-specific T cell responses measured by the IFNγ-ELISPOT assay at different time points postinfection (pi). At 38 and 61 weeks postinfection, PBMC from the same monkey were stimulated as described above. Spot Forming Cells (SFC) per 10^6 cells were evaluated in the presence of Babe-Gag BLCL (grey bars) and Babe-CTR BLCL (open bars). Error bars represent the standard deviation of duplicate samples.

chromium release assay detected specific lysis of Babe-Gag BLCL by CTL generated from PBMC of the SHIV89.6P-infected monkey at two different time points after infection. Specifically, at week 16 postinfection-specific lysis of target cells was low (12%), but at 20 weeks postinfection, it increased up to 60% at the same effector:target ratio (25:1) (Fig. 2A and Table 2). At 38 and 61 weeks postinfection, no specific lysis of target cells was detected by the chromium release assay (data not shown). However, at these time points, a SIV Gag-specific T cell response was detected using the IFNγ-ELISPOT assay described by Bercovici et al. (2000) (Fig. 2B and Table 2). Unfortunately, following this latest time point, Mk 760 showed disease progression, with high levels of plasmaviremia, and AIDS symptoms. For this reason, we decided to perform the next set of experiments on a different SHIV89.6P-infected monkey (Mk 783), which was able to control viral replication. Moreover, based on the higher expression of Gag and Env proteins (Table 1 and data not shown), FB-Gag and BAF-Env retroviral vectors were used to infect BLCL in order to evaluate the presence of antigen-specific T cells at different time points from week 80 up to week 110 postchallenge. Each experiment was replicated at least twice at different time points and the ranges of values (both percentage of lysis and SFC/10^6 cells) are reported in Table 2.

3.3. Comparison with “rVV method”

In order to compare the efficiency of our method with the widely used “rVV method”, CTL activity was measured in a ^{51}Cr release assay using rVV-Gag-infected and FB-Gag BLCL as both stimulator and target cells. Both cell lines were tested for Gag expression at the time of the experiment; notably, rVV-Gag BLCL always expressed higher levels of p27 than FB-Gag BLCL (Table 1). Fig. 3A shows a representative experiment in which the percentage of specific lysis was higher at every E:T ratio when Retro-BLCL were used. Remarkably, the lysis of control BLCL (rVV-βGal or FB-CTR BLCL) was significantly lower in the case of Retro-BLCL, showing low levels of nonantigen-specific T cell expansion. The IFNγ ELISPOT assay confirmed these results (Fig. 3B and Table 2). Indeed, stimulation with FB-Gag BLCL resulted in 4080 SFC × 10^6 cells, with low backgrounds (80 SFC × 10^6 cells, 51-fold ratio between SFC in the presence of specific and control target cells), whereas rVV-Gag BLCL induced lower expansion of antigen-specific IFNγ producing cells

Table 2
Ranges of values (percentage of lysis or SFC/10^6 cells) obtained by using BLCL infected with rVV or stably transduced with retroviruses expressing the indicated antigen in ^{51}Cr release and ELISPOT assays, performed at different time points from SHIV89.6P infection

^{51}Cr release assay (% of lysis)					ELISPOT assay
BLCL	Effector cells	E:T ratio			SFC/10^6 cells
		High[a]	Medium[b]	Low[c]	
Babe-Gag	total PBMC	87.4	22.8–74.5	13.8–48.1	400–680
Babe-CTR	total PBMC	33.3	9.9–22.8	2.2–18.1	100–120
rVV-Gag	total PBMC	60	43–48.7	33–38.4	1060–1660
rVV-βGal	total PBMC	34	28.9–31	21–21.2	70–360
FB-Gag	total PBMC	69.7–78	63.4–74	56.7–62	4080–7400
FB-Gag	$CD8^+$	71.5–81.2	66.7–69.7	56.5–56.8	5280–8540
FB-Gag	$CD4^+$	ND	5–9.8	ND	2640–3470
FB-CTR	total PBMC[d]	6.7–19.6	5.4–14.7	1.3–3.1	80–560
BAF-ENV	total PBMC	56.3	53–55.3	42.2–48	1600–2500
BAF-ENV	$CD8^+$	ND	49–66.8	44.9–52.6	880–2500
BAF-ENV	$CD4^+$	ND	1–12.8	ND	110–440
BAF-CTR	total PBMC[d]	14.5	5.3–13.7	3.5–4.6	130–700

[a] From 70:1 to 30:1.
[b] From 25:1 to 10:1.
[c] < 10:1.
[d] Effector cells were either total PBMC, $CD8^+$ or $CD4^+$, depending on the experimental conditions.

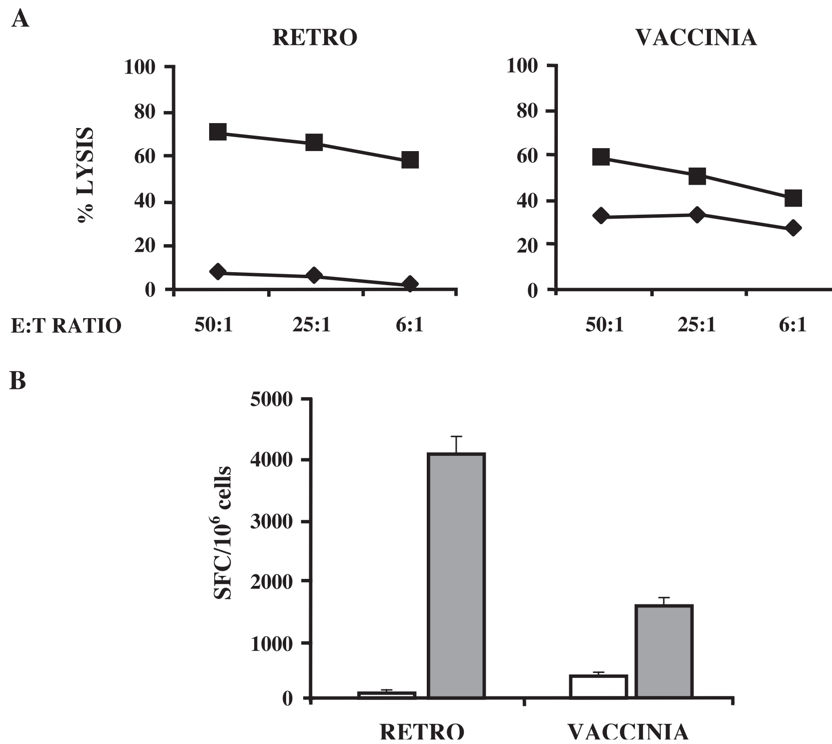

Fig. 3. Comparison of Gag-specific responses measured using FB-Gag BLCL or rVV-Gag BLCL. (A) CTL activity of PBMC from Mk 783 stimulated for 14 days with FB-Gag or rVV-Gag BLCL was measured in a standard ^{51}Cr release assay. The percentage specific lysis was determined by using the FB-Gag BLCL (■) or rVV-Gag BLCL (■) and the FB-CTR BLCL (♦) or rVV-βGal BLCL (♦), as target cells. (B) The effector cells stimulated as above described were tested for IFNγ production using the ELISPOT assay. The number of IFNγ producing T cells in the presence of FB-Gag BLCL (grey bars) or rVV-Gag BLCL (grey bars) and the FB-CTR BLCL (open bars) or rVV-βGal BLCL (open bars) is expressed as SFC/10^6 cells. Error bars represent the standard deviation of duplicate samples.

(1600 SFC/10^6 cells) with a much higher background (360 SFC/10^6, 4.6-fold ratio).

3.4. Induction of CD8$^+$ T cell expansion

To evaluate the phenotype of cells after 15 days of stimulation with the Retro-Gag transduced BLCL, experiments of CD4$^+$ cell depletion were performed. FACScan analysis showed a high percentage of CD8$^+$ T cells in the PBMC bulk population (about 80%) before the CD4$^+$ cell depletion, with a further enrichment after depletion of the CD4$^+$ T cells approaching 95% (data not shown). Subsequently, CTL activity and IFNγ production were evaluated in the bulk, CD8$^+$ and CD4$^+$ T cell populations. In addition, to confirm the effectiveness of this method, the presence of antigen-specific T cells vs. an additional antigen, SHIV89.6P Env, was analysed. Results obtained using FB-Gag BLCL and BAF-Env BLCL to stimulate specific CTL are shown in Figs. 4 and 5, respectively.

In a typical experiment shown in Fig. 4A, CD8$^+$ T cells generated similar if not higher levels of lysis of Gag-expressing target cells when compared to bulk effector cells. Conversely, CD4$^+$ effector T cells did not produce a significant specific lysis ($<10\%$) (Fig. 4A and Table 2). These results confirmed the ability of Retro-BLCL to induce expansion of antigen-specific CD8$^+$ T cells, able to recognize and lyse antigen-expressing target cells. Within the same experiment, the effector cell populations were also used to evaluate the production of IFNγ upon stimulation with FB-Gag BLCL. As shown in Fig. 4B, a high number of SFC (7400 vs. 80 SFC/10^6 cells in the presence of specific vs. control BLCL, respectively; 92-fold ratio) was found in the bulk population. A higher IFNγ production was detected in the CD8$^+$

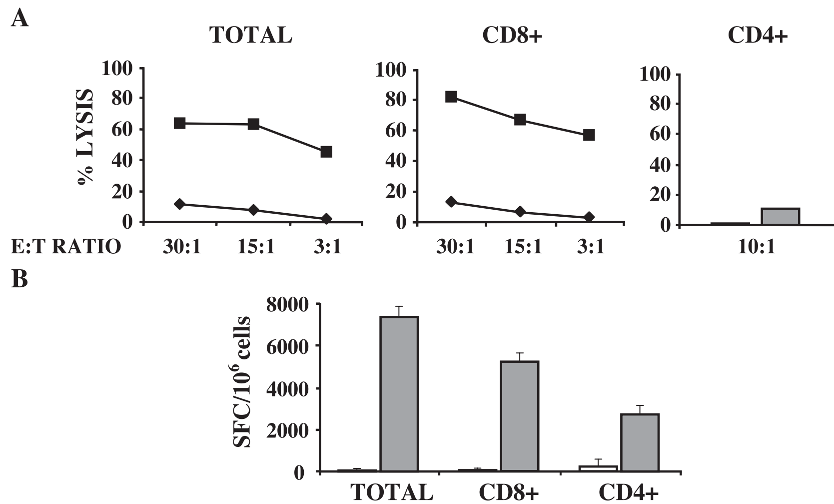

Fig. 4. Anti-SIV Gag-specific CTL activity (A) and IFNγ production (B) measured in Mk 783, infected with SHIV89.6P. PBMC from the infected monkey were stimulated for 14 days in the presence of autologous FB-Gag BLCL. Analysis of Gag-specific T cell responses in bulk population (total), $CD8^+$ and $CD4^+$ T cells. The percentage of specific lysis was determined by a standard ^{51}Cr release assay using the FB-Gag BLCL (■) or the FB-CTR BLCL (♦) as target cells. The number of IFNγ producing T cells (SFC/10^6 cells) was evaluated using the IFNγ ELISPOT assay, in the presence of FB-Gag BLCL (grey bars) and FB-CTR BLCL (open bars). Error bars represent the standard deviation of duplicate samples.

(5280 vs. 30 SFC/10^6 cells, 176-fold ratio) population rather than in $CD4^+$ T cells (2640 vs. 80 SFC/10^6 cells, 33-fold ratio). Interestingly, $CD4^+$ T cells showed a consistent IFNγ production, even if lower than $CD8^+$ T cells, in the absence of specific lysis. Moreover, the ratio between specific and nonspecific SFC/10^6 cells was much lower than that observed in the $CD8^+$ population.

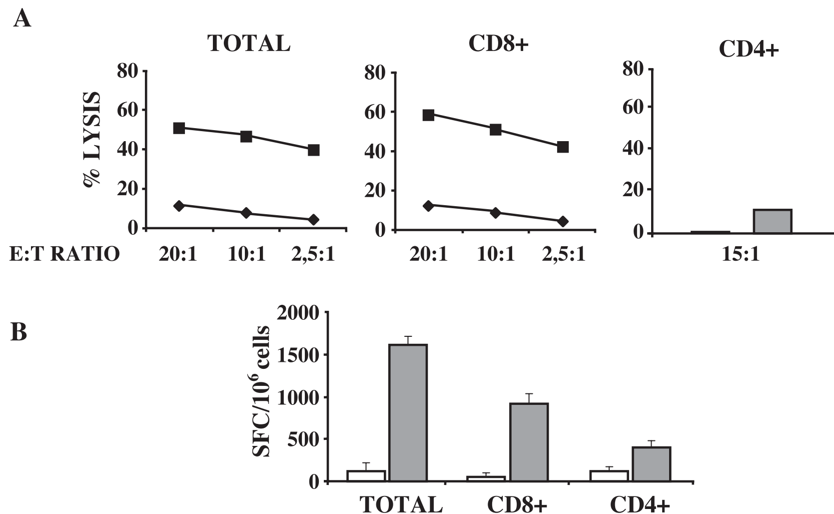

Fig. 5. Anti-HIV Env-specific CTL activity (A) and IFNγ production (B) measured in Mk 783, infected with SHIV89.6P. PBMC from the infected monkey were stimulated for 14 days in the presence of autologous BAF-Env BLCL. Analysis of Env-specific T cell responses in bulk population (total), $CD8^+$ and $CD4^+$ T cells. The percentage of specific lysis was determined by a standard ^{51}Cr release assay using the BAF-Env BLCL (■) or the BAF-CTR BLCL (♦), as target cells. The number of IFNγ producing T cells (SFC/10^6 cells) was evaluated using IFNγ ELISPOT assay, in the presence of BAF-Env BLCL (grey bars) and BAF-CTR BLCL (open bars). Error bars represent the standard deviation of duplicate samples.

To confirm these data, the same analysis was performed using effector cells stimulated with BLCL-expressing Env (BAF-Env BLCL). Fig. 5A shows a representative experiment in which the bulk PBMC population stimulated with BAF-Env BLCL was able to lyse the target cells. Yet again, the specific lysis of target cells obtained with the bulk population was due to $CD8^+$ T cells, which showed a higher specific lysis when compared to the bulk population (Fig. 5A and Table 2). As expected, $CD4^+$ effector cells did not produce a significant specific lysis at 15:1 E:T ratio. Results of IFNγ production (Fig. 5B and Table 2) confirmed the data obtained by using FB-Gag BLCL, suggesting the reproducibility of this method in the induction of an in vitro expansion of antigen-specific $CD8^+$ T cells.

4. Discussion

In the present report, we describe the use of an alternative method for the measurement of CTL activities in the *M. fascicularis* (cynomolgus monkey) animal model of AIDS, after infection with SHIV89.6P. It should be noted that this assay does not rely on the knowledge of MHC class I alleles from cynomolgus monkeys, which, to date, is not available. We have demonstrated that the use of BLCL transduced with retroviral vectors expressing SHIV89.6P antigens as stimulator and target cells was able to generate efficiently the in vitro expansion of antigen-specific $CD8^+$ T cells with cytolytic activity. Moreover, different retroviral vectors were constructed and used in order to evaluate which was the most efficient in expressing the antigen of interest in simian BLCL. Based on the expression of SIV-Gag protein, FB-Gag was more efficient than Babe-Gag and, in the same way, BAF-Env showed a higher production of HIV-Env protein compared to FB-Env and Babe-Env (Table 1 and data not shown).

To analyse the presence of CTL in different animal models as well as in humans, recombinant vaccinia viruses (rVV) are widely used to deliver viral antigens to stimulator/target cells in chromium release and IFNγ-ELISPOT assays (Rose et al., 2001; Sun et al., 2003). Here, we have shown that SIV-Gag-expressing Retro-BLCL exhibit greater lysis ability than rVV encoding SIV-Gag protein in spite of the higher expression of Gag protein in rVV-Gag-infected BLCL. In fact, the use of FB-Gag BLCL generated preferentially the expansion of specific $CD8^+$ effector T cells. Conversely, stimulation with rVV-Gag BLCL was less efficient (lower specific lysis), showing expansion of nonantigen-specific effector cells. The problem of the high background has already been described (Vogel et al., 1998; Nilsson et al., 2001; Sun et al., 2003) and one way possibly to reduce the vaccinia virus nonantigen-specific cytolysis present in bulk cultures would be to add an excess of cold targets in the test tube. The present results may indicate that in the rVV-Gag-infected BLCL, a larger array of peptides, derived not only from the recombinant protein of interest (SIV-Gag) but also from the rVV proteins, is potentially being presented at the MHC level. For this reason, a strong competition for presentation could occur among the SIV-Gag epitopes and the great amount of vaccinia virus antigens and this might decrease the frequency of Gag-specific CTL. This problem has been overcome using retroviral vectors expressing only the antigen of interest.

Throughout these experiments, we performed IFNγ ELISPOT and ^{51}Cr release assays on the same effector cells, in order to compare IFNγ production and the ability to kill specific target cells. When using bulk populations, different results were obtained. In the case of Mk 760, PBMC stimulated for 2 weeks with Retro-Gag BLCL showed IFNγ production at both 38 and 61 weeks postinfection, but not specific lysis of target cells. These results could be explained by the fact that the ELISPOT assay is more sensitive than ^{51}Cr release assay. Another possible explanation is that T cells from Mk 760 could have lost the ability to lyse target cells, due to disease progression, as described in other systems (Appay et al., 2000; Zhang et al., 2003, and reviewed in Letvin and Walker, 2003). On the other hand, after the acute phase of infection, Mk 783 was able to control viral replication throughout the course of the experiment. Indeed, in all of the assays performed, the IFNγ-producing bulk population was also able to kill the antigen-expressing target cells. After analysis of $CD8^+$ and $CD4^+$ purified populations, our data indicated that IFNγ-producing $CD8^+$ T cells had cytolytic activity, whereas the production of IFNγ from $CD4^+$ T cells was unrelated to the ability to lyse specific target cells. Because there is little evidence that $CD4^+$ T lymphocytes have

a major role as effector cells in HIV infection, these cells are most likely helping to facilitate in vivo antibody responses and CTL activity (reviewed in Letvin and Walker, 2003).

In conclusion, our results show that retrovirally transduced BLCL, expressing SHIV89.6P antigens, properly stimulate specific $CD8^+$ memory T cells in the absence of high levels of nonantigen-specific T cell expansion and represent a more efficient and specific method than that based on the use of rVV. In addition, this technology permits the establishment of permanent cell lines constitutively expressing the target antigen and overcomes the safety concerns inherent with handling a replication competent rVV. This, therefore, may represent an alternative tool for the analysis of $CD8^+$ T cell responses during vaccination protocols in those animal models where little information is available on MHC class I alleles or CTL epitopes.

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