

# Transduction of CpG DNA-Stimulated Primary Human B Cells with Bicistronic Lentivectors

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Recently, using HIV-1-derived lentivectors, we obtained efficient transduction of primary human B lymphocytes cocultured with murine EL-4 B5 thymoma cells, but not of isolated B cells activated by CD40 ligation. Coculture with a cell line is problematic for gene therapy applications or study of gene functions. We have now found that transduction of B cells in a system using CpG DNA was comparable to that in the EL-4 B5 system. A monocistronic vector with a CMV promoter gave  $32 \pm 4.7\%$  green fluorescent protein (GFP)<sup>+</sup> cells. A bicistronic vector, encoding IL-4 and GFP in the first and second cistrons, respectively, gave  $14.2 \pm 2.1\%$  GFP<sup>+</sup> cells and IL-4 secretion of  $1.3 \pm 0.2$  ng/10<sup>5</sup> B cells/24 h. This was similar to results obtained in CD34<sup>+</sup> cells using the elongation factor-1 $\alpha$  promoter. Activated memory and naive B cells were transducible. After transduction with a bicistronic vector encoding a viral FLIP molecule, vFLIP was detectable by FACS or Western blot in GFP<sup>+</sup>, but not in GFP<sup>-</sup>, B cells, and 57% of sorted GFP<sup>+</sup> B cells were protected against Fas ligand-induced cell death. This system should be useful for gene function research in primary B cells and development of gene therapies.

**Key Words:** HIV-1-derived lentivectors, bicistronic vectors, human primary B lymphocytes, CpG DNA, viral FLIP

## INTRODUCTION

Optimization of methods for gene delivery into primary human cells is important for research on gene functions and development of gene therapies [1]. Gene transfer into primary human B lymphocytes has been notoriously difficult. Successful applications of nonviral methods to functional assays of transgenes have not been reported for these cells. Epstein-Barr virus vectors so far have been tested only in B cell lines [2]. Murine oncoretroviral vectors gave low transduction efficiency (of up to about 4%) in primary human B cells; they could be utilized to study effects detectable by very sensitive methods, such as immunoglobulin (Ig) class-switch recombination detectable by PCR [3]. HIV-1-derived lentivectors gave efficient transduction of various immature and mature human hematopoietic cells [4–6]. Transgene expression restricted to B lymphocytes was obtained by grafting lentivector-transduced CD34<sup>+</sup> progenitors into NOD/SCID mice [7]. T lymphocytes could be transduced after activation, at least from the G0 to the G1 stage of the cell cycle [5,6]. But primary human B cells activated into

proliferation by crosslinking of CD40 in the presence of various cytokines were very poorly transducible [8,9]. Recently with such vectors we obtained efficient transduction of primary B cells cocultured with irradiated murine EL-4 B5 thymoma cells; monocistronic vectors with the human cytomegalovirus (CMV) or elongation factor-1 $\alpha$  (EF-1 $\alpha$ ) internal promoter gave  $27 \pm 12\%$  green fluorescent protein (GFP)<sup>+</sup> cells [9].

For gene therapy applications, however, a cell line potentially adds risks, such as generation of recombinant viruses. For studies of gene functions a handicap is that the functions of the thymoma cells have not yet been molecularly characterized. The first aim of this study was to find a culture system for transduction with HIV vectors of isolated B cells activated by defined stimuli. The LPS receptor, Toll-like receptor-4 (TLR4), is lacking in human B cells. But TLR9, the endosomal receptor for bacterial DNA (short single-stranded DNA containing nonmethylated CpG motifs; CpG DNA), is constitutively expressed in memory human B cells and rapidly upregulated by anti-Ig antibody—which mimics an antigen signal—in

naïve B cells [10,11]. We report here efficient lentiviral transduction of B cells cultured with CpG DNA, anti-Ig, IL-2, and IL-10.

The second aim was to test bicistronic HIV vectors encoding the transgene in the first cistron and GFP in the second cistron, downstream of the internal ribosomal entry site (IRES). The vectors contained the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) [12]. First, we tested a cytokine gene; this allows quantitation of secreted protein. IL-4 was chosen because it was produced neither by B cells nor in our CD34<sup>+</sup> cell-derived cultures. CD34<sup>+</sup> hematopoietic cells were utilized as a primary cell control known to be transducible with various HIV vector constructs [13,14]. As shown below, with the IL-4 vector using the CMV promoter the cytokine secretion rate of B cells was comparable to that of CD34<sup>+</sup>-derived cells transduced by this vector with the EF-1 $\alpha$  promoter. Second, we tested a viral FLIP gene. Viral FLIP proteins, like the short isoform of cellular FLIP, competitively replace the caspase in the proximal signaling complex that forms after the ligation of death receptors, such as Fas [15]. Thus, transduction should produce a positive (survival enhancing) effect rather than a loss of function readout only. Such an effect was obtained in Fas ligand (FasL)-exposed B cells.

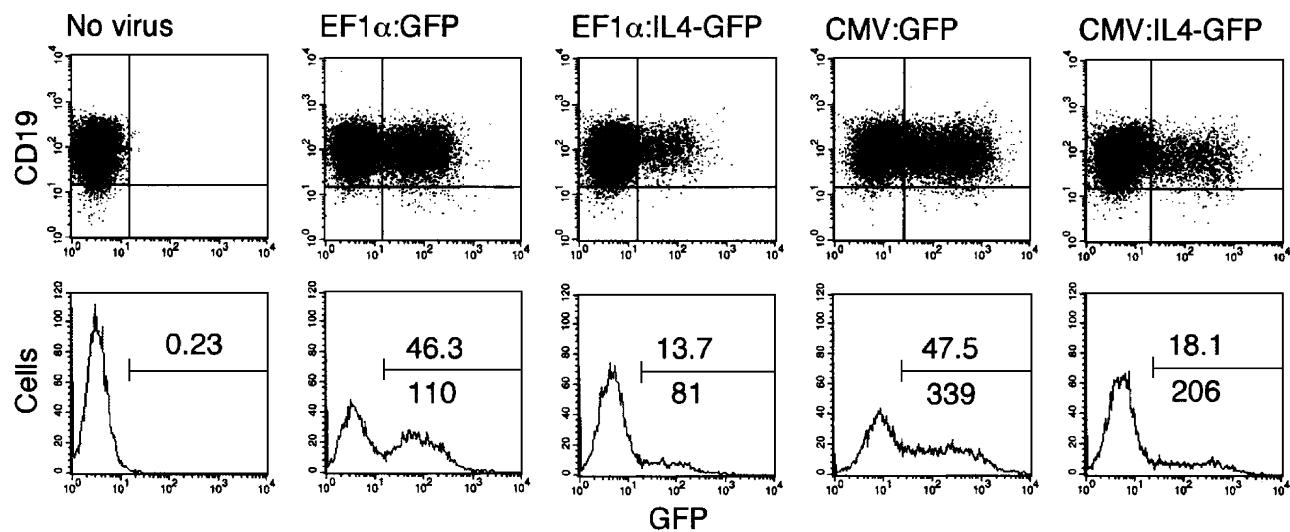
## RESULTS AND DISCUSSION

### B Cell Transduction in a Culture System Using CpG DNA

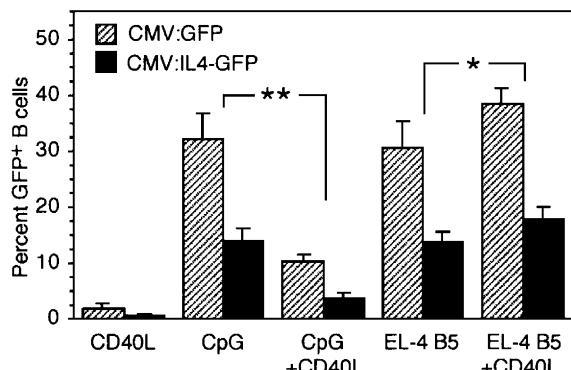
We found that peripheral blood B cells stimulated by CpG DNA, anti-Ig antibody, and the cytokines IL-2 and

IL-10, according to Bernasconi *et al.* [10], were efficiently transduced by HIV vectors. For monocistronic (GFP) and bicistronic (IL-4-IRES-GFP) vectors, the mean fluorescence intensity (MFI) of GFP<sup>+</sup> B cells was always higher (2.5-fold or more) with the CMV than with the EF-1 $\alpha$  internal promoter (Fig. 1), confirming our previous findings with monocistronic vectors [9]. As expected, the percentages of GFP<sup>+</sup> cells were lower with the bicistronic compared to the monocistronic vectors (3- or 2.3-fold by the mean, respectively, for the EF-1 $\alpha$  or CMV promoter; for the CMV promoter, mean data are shown in Fig. 2, CpG).

The CG-rich oligonucleotides C274 (21 bases) and 2006 (24 bases), which caused optimal B cell proliferation in the study by Marshall *et al.* [16], both allowed for efficient transduction, whereas oligonucleotide C661, with all CG inverted to GC, gave 20-fold lower proliferation and transduction. Vectors had to be added to the B cell cultures on day 2 or later. At a multiplicity of infection of 20 HeLa cell-transducing units per B cell (m.o.i. of 20) the numbers of GFP<sup>+</sup> cells were 1.2-fold higher by the mean than at an m.o.i. of 10, but at an m.o.i. of 30 or higher the numbers progressively decreased. We also found this both with vector particles added to culture plates by low-speed centrifugation at 1000g and with the particles left in the supernatant, whereas virus-free supernatant of the virus-producer cells showed no toxicity. In B cells, exposure to vector caused increase in annexin V<sup>+</sup> cells (on day 4, from 13% in control culture to 28% in culture with virus added on day 3 at an m.o.i. of 80), indicating that apoptosis was involved. Our vectors were not toxic for T cells and



**FIG. 1.** GFP MFI in B cells transduced with monocistronic or bicistronic HIV vectors was higher with the CMV than with the EF-1 $\alpha$  internal promoter. B cells were cultured in the CpG DNA system. Virus particles for the indicated vectors were added to parallel cultures on day 3 at an m.o.i. of 10. FACS analysis was done in viable cells on day 6. 2-D plots show staining with PE-anti-CD19 mAb on the y axis (isotype controls gave <0.5% background). Percentages of GFP<sup>+</sup> cells and MFI of these cells are indicated by upper and lower numbers, respectively, in the histograms.



**FIG. 2.** Differences in B cell transducibility by HIV vectors under various culture conditions. Vectors CMV:GFP or CMV:IL4-GFP were added on day 3 (m.o.i. of 10) to the CD40L system (CD40L), CpG DNA system (CpG), CpG DNA system in the presence of CD40L (CpG + CD40L), EL-4 B5 system (EL-4 B5), or EL-4 B5 system in the presence of CD40L (EL-4 B5 + CD40L). FACS analysis was done in viable cells on day 6 or 7. Shown are means  $\pm$  1 SEM for percentages of GFP<sup>+</sup> B cells from nine independent experiments for each culture condition. Transduction in CpG versus CpG + CD40L, and in EL-4 B5 versus EL-4 B5 + CD40L, was compared in parallel experiments. Significant, opposite effects of CD40L on transducibility were found with both vectors (\*\* $P$  < 0.001; \* $P$  < 0.05; by two-sided  $t$  test).

CD34<sup>+</sup> cells. This may reflect the fact that, in general, B cells are more prone to apoptosis in culture than many other cells.

#### Comparison of Different B Cell Culture Systems

Viable cell expansion in the CpG DNA system set up with total peripheral blood B cells was fivefold by the mean in 10 days. Individual B cells proliferate very heterogeneously in this system [10]. At least fivefold higher B cell expansion was obtained in the system using EL-4 B5 murine thymoma cells as helper T cells [9,17]. But the percentages of transduced B cells among the viable cells analyzed on day 6 or 7 were very similar in both systems (Fig. 2, CpG and EL-4 B5; there were about 30 and 14% GFP<sup>+</sup> cells, respectively, for vectors CMV:GFP and CMV:IL4-GFP). Since activated B cells proliferated and outnumbered nonproliferating cells, this means that proliferating B cells were similarly transduced in both systems. Various cell types, e.g., T cells [5,6], after some activation become transducible with lentivectors even if they do not divide. So far this could not be studied with B cells, because—unlike T cells—B cell populations could not be maintained in an activated, nondividing state.

In agreement with our previous data [9], however, transducibility was very low in B cells proliferating in response to soluble CD40 ligand, IL-2, and IL-10 (CD40L, Fig. 2). Moreover, when we added CD40L to the CpG DNA system (CpG + CD40L), thymidine incorporation was enhanced 2-fold compared to the standard CpG DNA system, but the transducibility became 3.2-fold lower by the mean ( $P$  < 0.001 by two-sided  $t$  test; nine independ-

ent, paired experiments). By contrast, adding CD40L to the EL-4 B5 system (EL-4 B5 + CD40L) gave not only slightly increased thymidine incorporation compared to the standard EL-4 B5 system, but also 1.3-fold higher B cell transducibility ( $P$  < 0.05; also nine independent, paired experiments). In those experiments in which the B response in the EL-4 B5 system was relatively weak, addition of CD40L could double the percentage of GFP<sup>+</sup> B cells. In addition we found that CD40L enhanced B transduction in total peripheral blood lymphocytes stimulated with pokeweed mitogen. Anti-Ig did not enhance transducibility in the CD40L system, but B cells stimulated with CpG DNA only were transducible (about 15% GFP<sup>+</sup> cells with monocistronic vector).

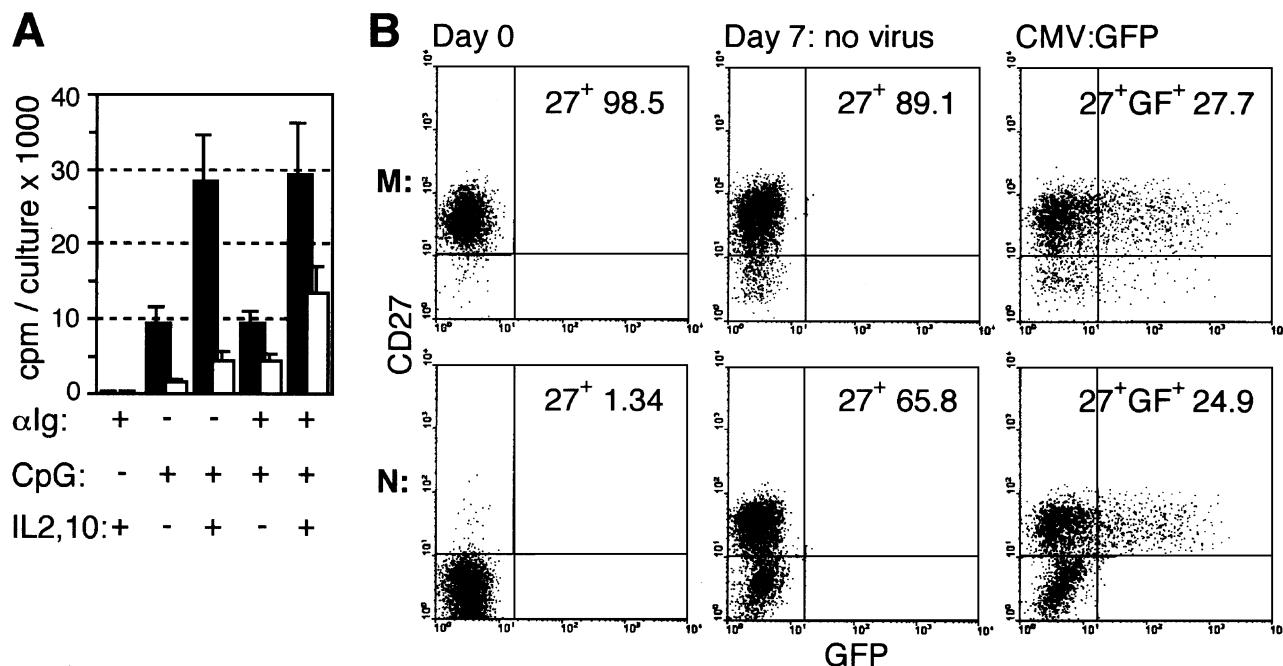
Thus, it appeared that, in particular, B cells stimulated via CD40 in the absence of other T cell signals (an artificial condition) somehow resisted transduction by HIV vectors. The vectors did not cause more annexin V expression in CD40L-stimulated than in CpG DNA-stimulated B cells. Serafini *et al.* [18] found that in B cells activated via CD40 the transduction was blocked at an early stage. It is possible that innate cellular defenses, such as mechanisms involving TRIM5 or APOBEC3 antiretroviral factors [19,20], are involved; they might be triggered under certain (abnormal?) cell activation conditions *in vivo*.

#### Transduction of Naive and Memory B Cells

In accordance with published data [10,11], anti-Ig enhanced the proliferation of naïve (CD27<sup>-</sup>), but not of memory (CD27<sup>+</sup>), B cells in the CpG DNA system (2.9-fold by the mean; Fig. 3A). Even with anti-Ig, proliferation was 2.1-fold lower in naïve than in memory B cultures. However, after 7 days of culture the majority of naïve B cells expressed CD27, and in cultures with vector these cells expressed GFP very similar to memory B cells (Fig. 3B shows data representative of two other independent experiments). CD27 is an activation marker for naïve B cells [21]. The naïve B cells that remained CD27<sup>-</sup> were not transduced. On the other hand, cultures set up with memory B cells always contained some CD27<sup>-</sup> cells after culture, but these cells were transduced. Since some B cells express CD27 ligand [21], these may have been cells with ligand-modulated CD27. In any case, the data show that activated naïve and memory B cells were transducible in the CpG DNA system.

#### IL-4 Secretion by Transduced B Cells

B cells transduced with IL-4 vectors, after cell wash, released IL-4 during secondary culture. We studied whether pseudotransduction (PTD) played a role, i.e., any viral integration-independent effects, including passive transfer of transgene proteins by the virus particles (cells become exposed to numerous defective particles) [9]. Therefore, we tested viruses produced using a pack-



**FIG. 3.** Naive and memory B cells proliferated and were transducible in the CpG DNA system. (A) Anti-Ig antibody ( $\alpha$ Ig) enhanced thymidine incorporation by naive (CD27<sup>-</sup>, white columns), but not by memory (CD27<sup>+</sup>, black columns), B cells in the presence of CpG DNA, with or without IL-2 and IL-10 (means  $\pm$  1 SEM of counts per minute (cpm) from three independent experiments). (B) The 2-D plots show CD27 expression and FL1 fluorescence in sorted memory and naive B cells before (Day 0) and after culture in the CpG DNA system (Day 7), with no virus or with vector CMV:GFP added on day 3 at m.o.i. of 10. Percentages of CD27<sup>+</sup> cells (27<sup>+</sup>) and of GFP<sup>+</sup> cells among the CD27<sup>+</sup> cells (27<sup>+</sup>GF<sup>+</sup>) are indicated (experiment representative for three independent experiments). Only those naive B cells that became CD27<sup>+</sup> were transduced.

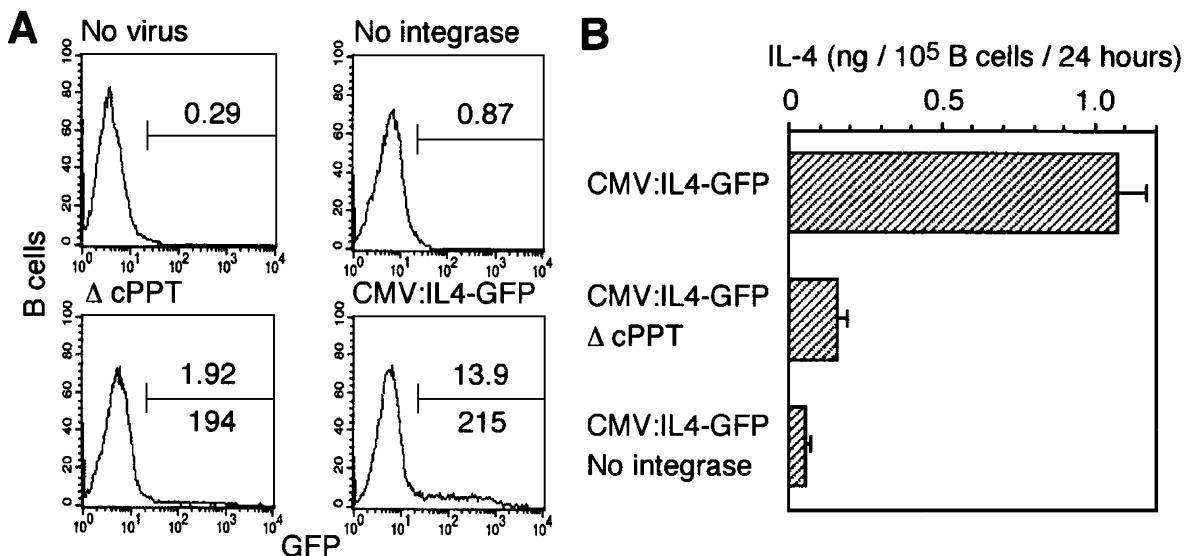
aging construct with a loss-of-function mutant integrase or a vector plasmid (CMV:IL4-GFP) lacking the central polypyrimidine tract (cPPT) involved in nuclear translocation [22]. We added control virus at an m.o.i. of 10 and the others, produced in parallel, at concentrations equalized for IL-4 concentrations in the 293T culture supernatant. Control vector gave 13.9% GFP<sup>+</sup> B cells (Fig. 4A) and an IL-4 release of 1.1 ng IL-4/10<sup>5</sup> B cells/24 h (Fig. 4B). The virus lacking integrase activity gave 0.87% GFP<sup>+</sup> cells, but no bright GFP<sup>+</sup> cells, i.e., this was weak PTD that occurs with GFP [9]. The IL-4 release was low, indicating that about 5% of the IL-4 release obtained with the positive control may have been due to PTD. The virus lacking the cPPT gave 1.92% GFP<sup>+</sup> cells, including about 1% bright cells. The IL-4 release (6.7-fold lower than control) was close to what one would expect for this number of bright GFP<sup>+</sup> cells plus PTD as estimated above. Thus, PTD played a negligible role.

#### Comparison of IL-4 Secretion by B Cells and CD34<sup>+</sup> Cells

We used CD34<sup>+</sup> cells as a reference for work with lentivectors. After transduction with the IL-4 vector with the EF-1 $\alpha$  promoter, which is optimal in these cells [13], the GFP expression in CD34<sup>+</sup> cell-derived cultures (Fig. 5A; the upper histograms) was similar to that obtained in B

cells with vector CMV:IL4-GFP. In CD34<sup>+</sup>-derived cells, intracytoplasmic IL-4 was detectable by FACS and correlated with GFP expression among individual transduced cells (lower histograms in Fig. 5A). Although B cells do not produce IL-4 [23], a strong nonspecific staining by the anti-IL-4 antibody in transduced and nontransduced B cells made such detection impossible. IL-4 secretion was also similar in B cells and CD34<sup>+</sup>-derived cells (Fig. 5B; about 1.2 ng IL-4/10<sup>5</sup> cells/24 h). In B cells, IL-4 secretion with the EF-1 $\alpha$  promoter was 3.4-fold lower than with the CMV promoter. In FACS-sorted GFP<sup>+</sup> B cells, the IL-4 secretion increased in parallel with their enrichment (not shown). Thus, transducibility with bicistronic vector was similar for B and CD34<sup>+</sup> cells. That many B cells become secretory cells (plasma cells) was apparently not relevant for the cytokine secretion rate. However, a considerably lower cytokine secretion rate was reported for primary B leukemia cells transduced with a bicistronic HIV vector lacking the WPRE [24]. This element enhances mRNA stability [12].

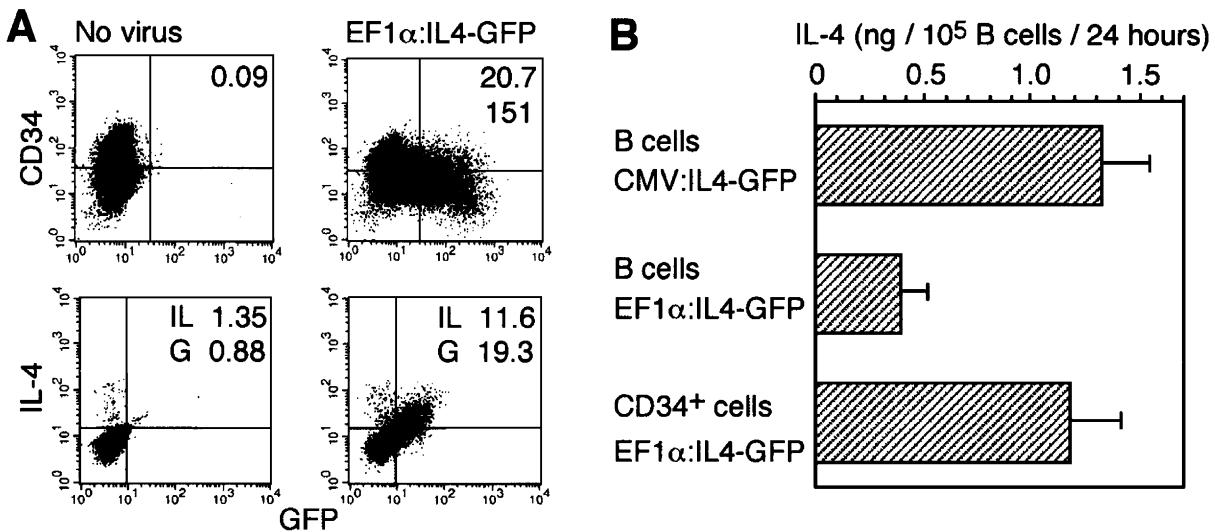
GFP expression and IL-4 secretion were stable in B cells between days 3 and 8 after exposure to vector and in CD34<sup>+</sup>-derived cells when followed up for 21 days. On day 8 after exposure to vectors with the respective optimal promoters, at an m.o.i. of 10, quantitative PCR revealed multiple copies of GFP DNA per diploid genome



**FIG. 4.** Correlation between IL-4 secretion and GFP expression in B cells exposed to different vector particles: positive control vector (CMV:IL4-GFP), vector plasmid deleted of the cPPT element ( $\Delta$  cPPT), and virus made using a packaging construct with an inactive mutant integrase (No integrase). (A) GFP expression. All three types of vector particles were produced in parallel. Control vector CMV:IL4-GFP was added at an m.o.i. of 10. The other vector particles were added at concentrations equalized for the amounts of IL-4 measured in the supernatants of the virus-producer cells. Upper and lower numbers represent percentages of GFP<sup>+</sup> cells and MFI, respectively. (B) IL-4 secretion rates measured in this representative experiment (means  $\pm$  1 SEM of triplicate cultures).

in CD34<sup>+</sup>-derived GFP<sup>+</sup> cells ( $4.5 \pm 0.3$ ), as expected [25], and also in CD19<sup>+</sup>GFP<sup>+</sup> B cells ( $4.8 \pm 0.4$ ; means  $\pm$  1 SEM,  $n = 4$ , i.e., two transduction experiments with mono- and two with bicistronic vectors for each cell type and for B cells one of each experiment in the CpG and B5 systems).

We obtained overlapping results with mono- and bicistronic vectors. We found at least 10-fold lower PCR signals in GFP<sup>-</sup> cells, showing low contamination by DNA plasmids. Since vector-derived episomal DNA is short-lived in various cells, including B cells [18], these



**FIG. 5.** GFP expression and IL-4 secretion by B cells transduced with vector CMV:IL4-GFP were similar to those found in cell cultures derived from cord blood CD34<sup>+</sup> cells transduced with vector EF1 $\alpha$ :IL4-GFP. (A) The upper plots show GFP expression in cell culture derived from 97% pure cord blood CD34<sup>+</sup> cells from a representative experiment. EF1 $\alpha$ :IL4-GFP was added on day 1 at an m.o.i. of 20; cells were analyzed on day 5. Upper and lower numbers represent percentages of GFP<sup>+</sup> cells and MFI, respectively. The lower plots show a staining with PE-anti-IL-4 mAb on the y axis and GFP fluorescence on the x axis on day 5. Percentages of cells stained by anti-IL-4 ("IL") and of GFP<sup>+</sup> cells ("G") are indicated. (B) IL-4 secretion rates measured in B cells and CD34<sup>+</sup> cell-derived cultures transduced with the indicated vectors at an m.o.i. of 10 (means  $\pm$  1 SEM from six independent experiments).

data indicate multiple vector integration in both cell types.

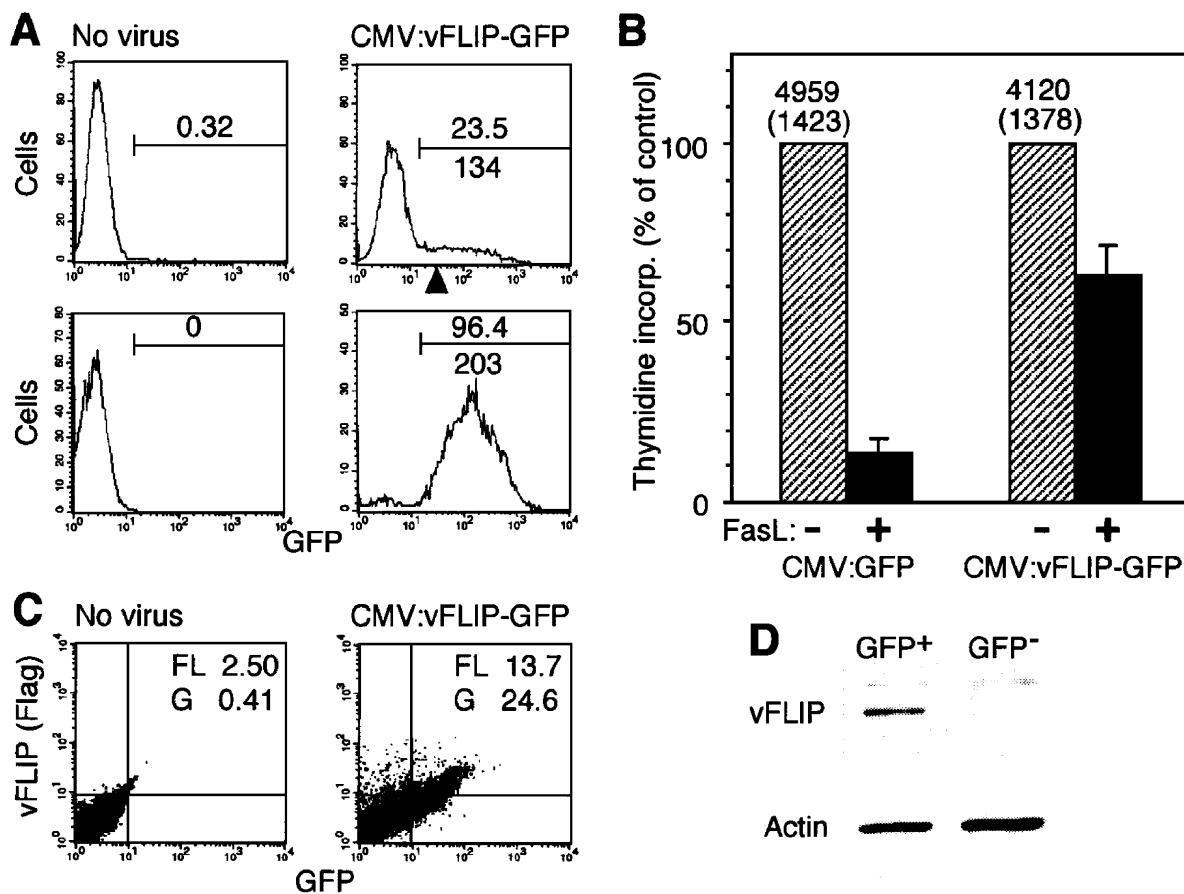
#### Effects of Bicistronic Vector Expressing Viral FLIP

We then tested a bicistronic vector expressing FLAG-tagged vFLIP of molluscum contagiosum virus [15]. We added this vector (CMV:vFLIP-GFP) or control vector (CMV:GFP) to B cells in the CpG DNA system on day 2 and sorted GFP<sup>+</sup> cells on day 5 (Fig. 6A). We recultured the cells in the presence or absence of soluble FasL. In two experiments, transduction of vFLIP caused a 1.8- to 2.4-fold reduction in the number of FasL-induced annexin V<sup>+</sup> apoptotic cells after 20 h (without and with FasL, respectively, there were 32 ± 3.9 and 79 ± 5.6% annexin V<sup>+</sup> cells for control vector versus 35 ± 4.4 and 57 ± 1.5 for

vFLIP vector). In three other experiments, we measured thymidine incorporation after 48 h to detect cell survival (Fig. 6B). Only 14 ± 4% of B cells transduced with control vector, but 63 ± 12% of B cells transduced with vFLIP vector, resisted killing by FasL. Thus, among those cells that could be killed by FasL, 57% (range 45 to 71%) were protected by vFLIP.

In B cells transduced in the EL-4 B5 system, intracellular vFLIP was detectable with anti-FLAG mAb by FACS and its expression correlated with GFP expression in individual cells (Fig. 6C). Viral FLIP was also detectable with this antibody by Western blot in sorted GFP<sup>+</sup>, but not in GFP<sup>-</sup>, B cells (Fig. 6D).

In conclusion, with an HIV-1-derived bicistronic vector we obtained proof of principle for the testing of



**FIG. 6.** Detection of viral FLIP protein and of protection from FasL-induced cell death in B cells transduced with vector CMV:vFLIP-GFP. (A) GFP expression in B cells transduced in the CpG DNA system with CMV:vFLIP-GFP at an m.o.i. of 10, before and after sorting of GFP<sup>+</sup> B cells. Upper and lower numbers represent percentages of GFP<sup>+</sup> cells and MFI, respectively. The gating for the FACS sorting is shown by the arrowhead. (B) Thymidine incorporation by sorted GFP<sup>+</sup> B cells transduced with CMV:vFLIP-GFP, or control vector CMV:GFP, after reculture in the presence or absence of FasL. The black columns represent percentages of counts per minute (cpm) relative to no-FasL controls (means ± 1 SEM from three independent experiments). Mean cpm in cultures with no FasL are indicated (SEM in parentheses). (C) Detection of intracellular viral FLIP with anti-FLAG mAb (y axis) and GFP fluorescence (x axis) in B cells transduced with CMV:vFLIP-GFP in the EL-4 B5 system. Vector was added on day 3 at an m.o.i. of 10 and cells were analyzed on day 8, after 46-fold cell expansion. Percentages of cells stained by anti-FLAG ("FL") and of GFP<sup>+</sup> cells ("G") are indicated. (D) Western blot of sorted GFP<sup>+</sup> and GFP<sup>-</sup> B (CD19<sup>+</sup>) cells transduced as above in the EL-4 B5 system, performed with protein equivalents of  $3 \times 10^5$  B cells. Stainings with anti-FLAG mAb or anti-actin antibodies are shown. Viral FLIP was detected as a band of 34 kDa.

a gene function in primary human B cells. The CpG DNA culture system should be useful for such studies.

## MATERIALS AND METHODS

### HIV-1-Derived Vectors

A second-generation packaging system and self-inactivating HIV-1 vectors [26] with the pLoxP site in the 3'LTR [27] were utilized. The monocistronic vector with the EF-1 $\alpha$  internal promoter (called vector EF1 $\alpha$ :GFP in this study) was plasmid pWPT-GFP for which the map and sequence are available at <http://www.tronolab.unige.ch>. This plasmid carries, in the 5' to 3' direction, the cPPT element [22], the intronless ("short") EF-1 $\alpha$  promoter, the expression cassette, and the WPRE [12]. Vector CMV:GFP was made by replacing the promoter with the human CMV promoter [13] between the *Cla*I and the *Bam*HI restriction sites. In the bicistronic vectors, which were derived from the above vectors, the first cistron was the transgene of interest, between the *Bam*HI and the *Sal*I restriction sites, followed by the encephalomyocarditis virus IRES [27] and GFP encoded downstream of the IRES. In the vectors EF1 $\alpha$ :IL4-GFP and CMV:IL4-GFP the transgene was the complete human IL-4 cDNA sequence (a kind gift from Dr. J.-F. Gauchat, formerly at the Institut Pierre Fabre, St. Julien, France). In vector CMV:vFLIP-GFP the transgene was the FLAG-tagged viral FLIP of human molluscum contagiosum virus (open reading frame 159L), kindly provided by Dr. Margot Thome of the Department of Biochemistry, University of Lausanne [15]. In some experiments we used a vector plasmid as above, except that it lacked the cPPT element, or a packaging construct [9] with a loss-of-function mutant integrase (mutation D64V) [28].

Virus particles pseudotyped with vesicular stomatitis virus G glycoprotein were produced, concentrated 100-fold by ultracentrifugation, and titrated on HeLa cells by measurement of GFP $^+$  cells by FACS to establish HeLa transducing units per milliliter (usually 0.5 to 2  $\times$  10 $^8$ ), as described [9]. Viral stocks were suspended in B cell culture medium and stored at -70°C.

### Cell Cultures and Transduction

Approval for research on human cells was obtained from the Ethics Committee of the Geneva University Hospitals. Written consent was provided for all cell samples. B cells were isolated to more than 99% purity from buffy coats of virus-screened blood donations obtained from the Geneva Transfusion Center using Ficoll-Hypaque centrifugation and anti-CD19 magnetic beads (Dynal) [29]. All B cell cultures were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 2-mercaptoethanol, Hepes buffer, and antibiotics as described [29].

**CpG DNA system.** B cells (25,000 cells/200  $\mu$ l culture; flat-bottom 96-well plates) were stimulated with CpG DNA (2.5  $\mu$ g/ml), anti-Ig antibody (2  $\mu$ g/ml), IL-2 (50 ng/ml), and IL-10 (10 ng/ml). CpG oligonucleotides of the published sequences C274, 2006, and C661 [16] were obtained as complete phosphorothioates synthesized to our order by Microsynth (Balgach, Switzerland). Anti-Ig was goat F(ab) $_2$  anti-human IgA + IgG + IgM (H+L) (Jackson ImmunoResearch). The cytokines were from PeproTech. FACS sorting of CD27 $^+$ /CD27 $^-$  cells was done as described [29].

**CD40L system.** B cells (50,000 cells/200  $\mu$ l) were stimulated with a soluble oligomeric CD40L (300 ng/ml), IL-2 (50 ng/ml), and IL-10 (10 ng/ml) as described [29]. When CD40L was added to the CpG DNA system or the EL-4 B5 system, its concentration was also 300 ng/ml.

**EL-4 B5 system.** B cells (3000 cells/200  $\mu$ l) were stimulated with 50,000 irradiated EL-4 B5 murine thymoma cells and a mixture of human cytokines acting on the EL-4 B5 cells (IL-1 $\beta$ ) or the B cells (TNF- $\alpha$ , IL-2, and IL-10), as described [9].

To transduce B cell cultures at different times, the big cell clusters, which formed in all culture systems, were dispersed by gentle pipetting (five times aspiration of the culture using 200- $\mu$ l tips). Virus particles were then added to the cultures in a volume of 2 to 4  $\mu$ l, at the indicated m.o.i. according to cell counts by microscope (B cells or B plus EL-4 B5 cells),

without further manipulations or cell wash. We found that centrifugation of virus particles in new culture plates (spinoculation) with cell transfer did not give better results.

**CD34 $^+$  cell culture and transduction.** Umbilical cord blood CD34 $^+$  cells were isolated to >95% purity with anti-CD34 magnetic beads (Dynal), cultured, and transduced as described [13]. Briefly, the cells (10 $^5$ /200  $\mu$ l) were first cultured and transduced on day 1 in the presence of thrombopoietin (TPO). After 48 h, the cells were recultured in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum, antibiotics, TPO, Flt3-ligand, and stem cell factor; the cells were recultured every 6 days.

### Sorting and FACS Analysis of Transduced Cells

Various GFP $^+$  cell populations were sorted on a FACStar sorter, and analyses of GFP $^+$  cells were performed on a FACScan analyzer using CellQuest software (Becton-Dickinson), at the cytofluorometry laboratory of the Geneva Medical Center. Cells were pretreated with polyclonal mouse Ig before staining with phycoerythrin (PE)-coupled anti-CD19 [9], anti-CD27 [29], or anti-CD34 [13] mAb, and viable (7-amino-actinomycin-D low) cells were gated for analysis of GFP expression [9]. For analysis of cytoplasmic IL-4, cells were cultured with brefeldin A (Sigma), 2.5  $\mu$ g/ml, for 3 h. For analysis of cytoplasmic IL-4 or viral FLIP, cells were treated with Cytofix/Cytoperm and Perm/Wash solutions, followed by AB $^+$  serum, and then stained, respectively, with PE-coupled anti-IL-4 mAb (Pharmingen) or anti-FLAG-M2 mAb (Sigma), which we coupled to biotin, followed by PE-coupled streptavidin (Jackson ImmunoResearch). The isotype control was mouse IgG1. Apoptosis was assessed using the Annexin V-PE Apoptosis Detection Kit I (Becton-Dickinson).

### Other Analyses of Transduced Cells

**IL-4 secretion rate.** On day 2 or 3 after addition of viral particles, B cells or CD34 $^+$  cells were harvested and washed twice in culture medium in Eppendorf tubes, and viable, trypan blue-excluding cells were counted. The cells were recultured as before, and after 24 h soluble IL-4 was measured in culture supernatants by Quantikine Human IL-4 Immunoassay (R&D Systems).

**Real-time quantitative PCR.** The LightCycler system, High Pure PCR Template Preparation Kit, and LightCycler FastStart DNA Master<sup>PLUS</sup> SYBR Green I kit (all from Roche) were utilized. Cells were transduced with vectors treated with DNase I before ultracentrifugation [7]. PCR was performed in separate assays for genomic DNA, using intron-binding primers for  $\beta$ 2-microglobulin (5'-GGCACTGCTGAGATACTGAT-3', reverse 5'-GCTAGGACAGCAGGACTTA-3'; 215-bp product) and for vector DNA, using GFP primers (5'-GGCAAGCTGACCCTGAAGTT-3', 5'-GCATGGCGGACTTGAAGAAGT-3'; 149-bp product); respective plasmid standards were utilized.

**FasL assay.** GFP $^+$  cells were sorted 3 days after exposure to vector and recultured (20,000 trypan blue-excluding cells/200  $\mu$ l, triplicate cultures) in the CpG DNA system (without anti-Ig) in the presence of soluble oligomeric FasL (200 ng/ml) prepared as described [30]. Thymidine incorporation was measured after 48 h including the pulsing during the last 12 h [29] with 1  $\mu$ Ci (0.037 MBq) [ $methyl$ -<sup>3</sup>H]thymidine (Amersham Pharmacia Biotech).

**Western blot for viral FLIP.** This was done with the ECL Western blotting analysis system (Amersham) using 0.45- $\mu$ m nitrocellulose membranes (Bio-Rad) and Biomax chemiluminescence films (Kodak). The antibodies were IgG1 mouse anti-FLAG-M2 mAb (Sigma) or polyclonal goat IgG anti-actin (Santa Cruz Biotechnologies) in conjunction with horseradish peroxidase-coupled anti-mouse or anti-goat antibodies, respectively (Santa Cruz Biotechnologies).

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