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Bystander Macrophages Silence Transgene Expression Driven by the Retroviral Long Terminal Repeat

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The Moloney murine leukemia virus (MLV)-based retroviral vector has been widely used for transfer of exogenous genes to various organs and tissues. Although the long terminal repeat (LTR) of MLV allows for transgene expression in a wide range of cell type, its activity is often silenced *in vivo*. In reporter macrophages transduced with a MLV-based retroviral vector, activity of the LTR was transiently and reversibly suppressed following stimulation by lipopolysaccharide (LPS). When unstimulated reporter macrophages were co-cultured with LPS-stimulated, untransduced macrophages, the LTR activity was similarly depressed. Activity of the LTR in retrovirus-transduced, mesangial cells was also down-regulated when co-cultured with activated macrophages. This suppressive effect was reproduced by cross-feeding with culture media conditioned by activated macrophages. LPS-stimulated macrophages abundantly expressed cytokines including IL-1β, tumor necrosis factor-α (TNF-α) and transforming growth factor-β1 (TGF-β1). When externally added, TNF-α and/or TGF-β1, but not IL-1β, depressed activity of the LTR in reporter macrophages and reporter mesangial cells. These results raise a possibility that expression of transgenes driven by the MLV-LTR may be silenced *in vivo* when the retrovirally-transduced cells are co-localized with activated macrophages.

Key Words: **gene transfer; Moloney murine leukemia virus; retrovirus; long terminal repeat; macrophage; cytokine.**

The Moloney murine leukemia virus (MLV)-based retroviral vector is a widely-used gene transfer vehicle for transduction of various tissues and organs (1). Despite its extensive use under a broad array of experimental and clinical settings, it is still unclear whether and how activity of the long terminal repeat (LTR) is affected in certain pathophysiologic circumstances. In general, viral promoters/enhancers contain binding sites for endogenous transcription factors. Altered activity of transduced host cells in response to environmental stimuli, possibly, affects activity of the retroviral LTR.

Transgene expression driven by the MLV-LTR is occasionally lost *in vitro* and *in vivo*. Several mechanisms have been proposed to date; e.g., immune-mediated elimination of transduced cells, suppression of transgene expression by endogenous repressor elements, instability of proviral DNA, and methylation of introduced gene sequences (1). However, currently, pathophysiological situations relevant to the *in vivo* silencing of the retroviral LTR are unknown. I found that, in macrophages transduced with a MLV-based retroviral vector, activity of the LTR was down-regulated following stimulation of the cells by lipopolysaccharide (LPS). It was associated with neither cell death nor deletion of the transgene sequence. The present investigation was initiated to elucidate mechanisms involved in this negative regulation of the MLV-LTR by activated macrophages.

MATERIALS AND METHODS

Cells. The normal alveolar macrophage cell line NR8383 (2) derived from a Sprague-Dawley rat was purchased from American Type Culture Collection (Manassas, VA). Rat mesangial cells were cultured from isolated renal glomeruli of a male Sprague-Dawley rat and identified as being of mesangial cell phenotype as described before (3). The reporter macrophage BAGMAC^{NR} and the reporter mesangial cell MLTRZ were established as described before (4,5). In brief, cells were exposed to diluted conditioned medium from a helper-free packaging cell line ΩE/BAG (5) that produces a replication-incompetent β-gal-at-gag (BAG) virus. This retroviral vector introduces a β-galactosidase gene (*lacZ*) and a neomycin phosphotransferase gene (*neo*) under the control of the MLV-LTR and the simian virus 40 (SV40) promoter, respectively (6). Stable infectants were selected in the presence of neomycin analogue G418 (500–750 μg/ml; Sigma Immunochemicals, St. Louis, MO), and the reporter cells were established.

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Pharmacological manipulation. Reporter cells were treated with 1 μg/ml LPS (*Escherichia coli* 0111:B4; Sigma Immunochemicals) for 24 -36 h and subjected to 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) assay and Northern blot analysis. To evaluate reversibility of altered promoter activity, LPS-stimulated BAGMAC^{NR} cells were washed three times, incubated in the absence of LPS for up to 7 days and subjected to Northern analysis. In some experiments, LPS-pretreated cells were cultured in the presence of the DNA-demethylating agent 5-azacytidine (1–10 μM; Sigma) for 24–48 h, and activity of the viral promoters was retested. Effects of cytokines on the activity of the MLV-LTR and the SV40 promoter was examined by exposing reporter cells to human IL-1β (10 ng/ml; Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan), human tumor necrosis factor-α (TNF-α; 100 U/ml; a gift from Dr. K. Noguchi, Tokyo) or human transforming growth factor-β1 (TGF-β1; 20 ng/ml; Genzyme, Cambridge, MA) for 24 h.

Co-culture. NR8383 macrophages were cultured in the presence or absence of LPS (0.5–1 μg/ml) for 12 h. After washing, LPS-stimulated and -unstimulated NR8383 cells were mixed with the same number of unstimulated BAGMAC^{NR} cells. After incubation for 24 h, cells were harvested together and used for Northern blot analysis. LPS-stimulated and -unstimulated NR8383 cells were also seeded on confluent cultures of MLTRZ mesangial cells. After incubation for 24 h, macrophages were removed, and MLTRZ cells were subjected to Northern analysis.

Cross-feeding. Macrophage-conditioned media were prepared as follows. NR8383 cells were seeded in 6-well plates at a density of 5 x 10^5 cells/well and stimulated with or without 1 μg/ml LPS. After 6 h, cells were washed three times and then incubated in 2.5 ml of 10% FCS medium for 24 h. The media were then collected, filtered and used for cross-feeding studies. MLTRZ mesangial cells were exposed to 1:1 diluted (50%) macrophage conditioned media for 24 h and used for Northern blot analysis.

X-gal assay. X-gal assay was performed as described before (4,5). In brief, fixed cells were incubated at 37°C for 10 min in the substrate solution containing 1 mg/ml X-gal (Sigma), 20 mM K3Fe(CN)6, 20 mM K4Fe(CN)6 · 3H2O, 2 mM MgCl2, 0.01% sodium desoxycholate and 0.02% Nonidet P-40 in PBS (pH 7.4). The reaction was terminated using 10% formaldehyde.

Northern blot analysis. Total RNA was extracted by a single-step method and subjected to Northern blot analysis, as described before (7). cDNAs for *lacZ* (6), *neo* (6), IL-1β (8), TNF-α (9) and TGF-β1 (10) were used as probes. As a loading control, expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used.

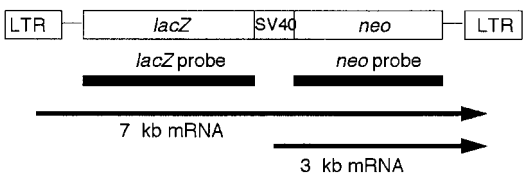

FIG. 1. Structure of the β-gal-at-gag (BAG) retroviral vector. The BAG retroviral vector encodes a bacterial β-galactosidase gene (*lacZ*) and a neomycin phosphotransferase gene (*neo*). The long terminal repeat (LTR) of Moloney murine leukemia virus (MLV) drives the expression of *lacZ*, and the simian virus 40 (SV40) early promoter drives the expression of *neo*. Transduced cells express a 7 kb mRNA which is detectable by either a *lacZ* probe or a *neo* probe. This large transcript is the *lacZ-neo* fusion mRNA. When probed by *neo*, duplex mRNAs, 7 kb and 3 kb, can be detected.

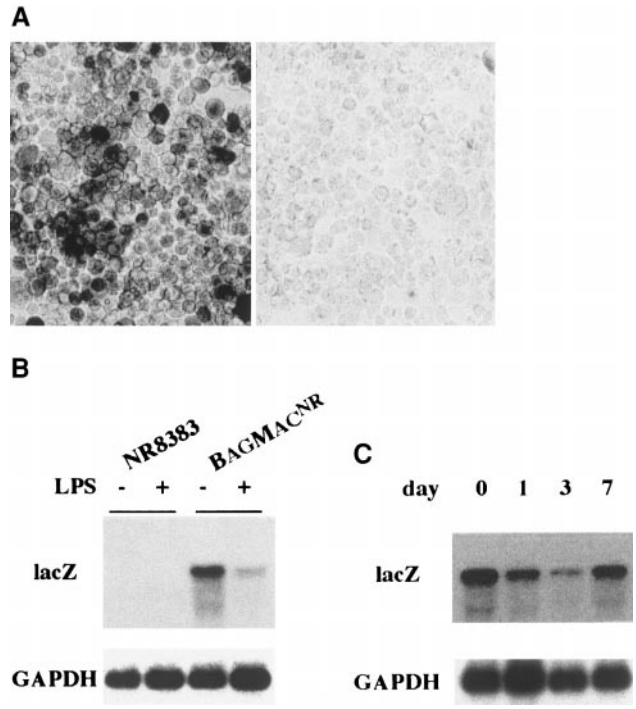

FIG. 2. Inactivation of the MLV-LTR in BAGMAC^{NR} macrophages stimulated by lipopolysaccharide (LPS). Reporter macrophage BAGMAC^{NR} was established by transduction of normal rat NR8383 macrophages with BAG virus. **A.** Activity of β-galactosidase in BAGMAC^{NR} macrophages. BAGMAC^{NR} cells were stimulated with (right) or without (left) 1 μg/ml LPS for 36 h, and β-galactosidase expression was examined by 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) assay. **B.** Expression of *lacZ* in BAGMAC^{NR} macrophages. NR8383 cells and BAGMAC^{NR} cells were stimulated with (+) or without (−) LPS for 24 h and subjected to Northern blot analysis. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as a loading control. **C.** Reversibility of the depressed LTR activity. BAGMAC^{NR} cells stimulated by LPS for 14 h were washed repeatedly and incubated in the absence of LPS for up to 7 days. After 1, 3 and 7 days, expression of *lacZ* was examined by Northern blot analysis.

RESULTS

Suppression of the MLV-LTR in activated macrophages. Reporter macrophage BAGMAC^{NR} was established by transduction of normal rat NR8383 macrophages with the replication-incompetent BAG virus, as described before (4). This retroviral vector introduces *lacZ* and *neo* under the control of the MLV-LTR and the SV40 early promoter, respectively (Fig. 1). The established BAGMAC^{NR} cells exhibited high levels of *lacZ* expression and β-galactosidase activity when examined by Northern blot analysis and X-gal assay (4).

Expression of β-galactosidase driven by the MLV-LTR was examined before and after stimulation of BAGMAC^{NR} cells by LPS. X-gal assay revealed that β-galactosidase activity was dramatically suppressed after stimulation of the cells by LPS (Fig. 2A). It was further confirmed by Northern blot analysis. Compared to unstimulated BAGMAC^{NR} cells, the cells stim-

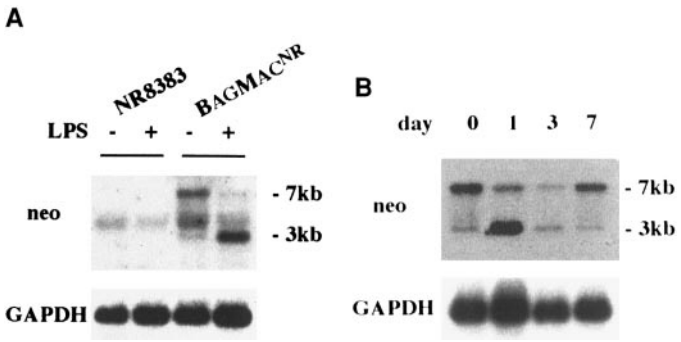

FIG. 3. Opposite kinetics of the MLV-LTR and the SV40 promoter in LPS-stimulated BAGMAC^{NR} macrophages. **A.** Opposite regulation of 7 kb and 3 kb *neo* transcripts. Parental NR8383 macrophages and BAGMAC^{NR} cells were stimulated with (+) or without (−) LPS, and expression of the 7 kb transcript (driven by the MLV-LTR) and the 3 kb transcript (driven by the SV40 promoter) was examined by Northern blot analysis. **B.** Time-lapse study. LPS-stimulated BAGMAC^{NR} cells were washed repeatedly and incubated in the absence of LPS for up to 7 days. After 1, 3 and 7 days, expression of *neo* was examined by Northern analysis.

ulated by LPS showed depression of the *lacZ* mRNA (Fig. 2B). To examine whether the attenuated activity of the LTR is reversible, LPS-prestimulated BAGMAC^{NR} cells were cultured in the absence of LPS. Northern blot analysis showed that the depressed *lacZ* expression was spontaneously recovered within 7 days (Fig. 2C).

BAGMAC^{NR} cells express a 7 kb transgene transcript which is detectable by a *lacZ* probe (4). This large transcript is a *lacZ-neo* fusion mRNA driven by the MLV-LTR. When probed by *neo*, the cells exhibit duplex mRNAs; approximately 7 kb and 3 kb (5). The former is driven by the MLV-LTR and the latter by the SV40 promoter (Fig. 1). Using a *neo* probe, activity of the SV40 promoter and the MLV-LTR was examined in parallel in activated BAGMAC^{NR} cells. Consistent with the result noted above, the activity of LTR indicated by the 7 kb transcript was down-regulated in LPS-stimulated BAGMAC^{NR} cells. In contrast, activity of the SV40 promoter indicated by the 3 kb transcript was up-regulated in the LPS-triggered BAGMAC^{NR} macrophages (Fig. 3A). A time-lapse study showed opposite kinetics of the SV40 promoter and the LTR; i.e., following the LPS stimulation, activity of the LTR was attenuated at day 1 to day 3, whereas the activity of the SV40 promoter was transiently enhanced at day 1 (Fig. 3B). Importantly, the activity of the LTR was suppressed at day 3 when the activity of the SV40 promoter was returned to the basal level. These results suggested; i) inactivation of the LTR in stimulated macrophages was due to neither cell death nor deletion of the transgene sequences, ii) the MLV-LTR was selectively suppressed in activated macrophages, and iii) the suppression of the LTR was not due to *cis*-acting repression by the activated 3' promoter.

Bystander suppression of the MLV-LTR by activated macrophages. To examine whether activated, bystander macrophages affect LTR activity of neighboring cells, unstimulated BAGMAC^{NR} cells were co-cultured with parental NR8383 macrophages pretreated with or without LPS. Activity of the LTR in BAGMAC^{NR} macrophages was examined by Northern blot analysis. As shown in Fig. 4A, activity of the LTR probed by the 7 kb transcript was down-regulated by pre-activated, bystander NR8383 cells. In contrast, activity of the SV40 promoter probed by the 3 kb *neo* mRNA was up-regulated by co-culture with activated

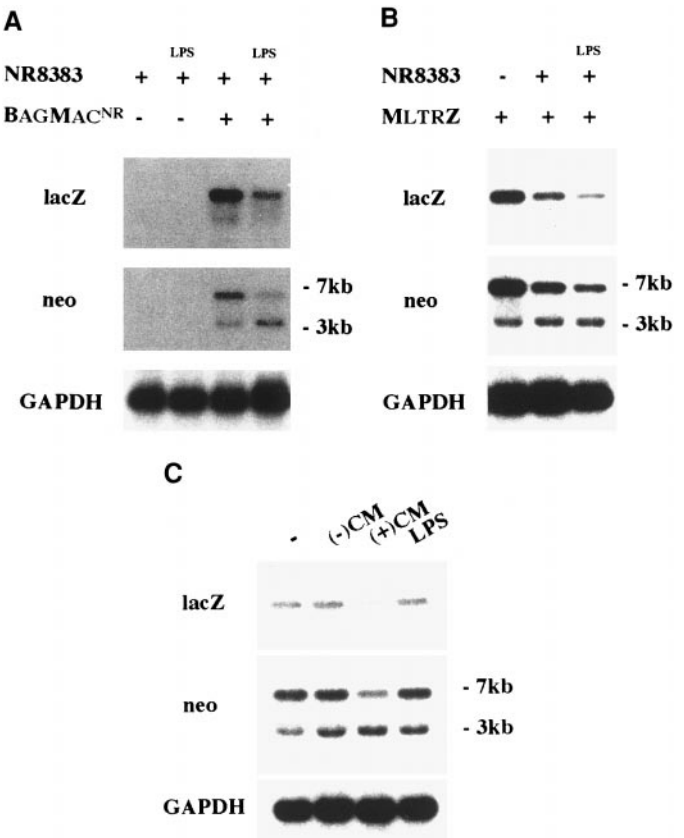

FIG. 4. Suppression of the MLV-LTR by activated, bystander macrophages. **A.** Co-culture of BAGMAC^{NR} macrophages with activated NR8383 cells. NR8383 macrophages were stimulated with or without LPS (0.5 μg/ml) for 12 h. After washing repeatedly, these cells were mixed with the same number of unstimulated BAGMAC^{NR} cells and seeded on plastic. After 24 h, cells were harvested together and subjected to Northern blot analysis of *lacZ* and *neo*. **B.** Co-culture of MLTRZ mesangial cells with activated NR8383 cells. LPS-stimulated and -unstimulated NR8383 cells were seeded on confluent cultures of the BAG-transduced, MLTRZ mesangial cells. After incubation for 24 h, NR8383 cells were removed, and MLTRZ cells were subjected to Northern analysis. **C.** Cross-feeding. Macrophage-conditioned media were prepared as described in Materials and Methods. MLTRZ mesangial cells were incubated for 24 h in the presence of 1:1 diluted (50%) conditioned media, and Northern blot analysis was performed. − : unconditioned medium, (−)CM: medium conditioned by unstimulated NR8383 cells, (+)CM: medium conditioned by LPS-pretreated NR8383 cells, LPS: 1 μg/ml.

NR8383 cells. The bystander effect by activated macrophages was further investigated using BAG virus-transduced mesangial cells. Reporter mesangial cell MLTRZ (11) was co-incubated with NR8383 macrophages pretreated with or without LPS. Consistent with the result noted above, co-culture of MLTRZ cells with activated macrophages led to suppression of the MLV-LTR activity (Fig. 4B, *lacZ* and 7 kb *neo*). Co-culture with unstimulated NR8383 cells also modestly reduced the activity of the LTR.

To elucidate mechanisms involved in the bystander action of activated macrophages, cross-feeding studies were performed. Conditioned media were prepared using LPS-stimulated and -unstimulated NR8383 macrophages, and MLTRZ cells were exposed to the diluted conditioned media. Northern blot analysis showed that the medium derived from activated macrophages depressed the LTR-driven transgene expression (Fig. 4C). In contrast, the SV40 promoter-driven *neo* expression was up-regulated by the macrophage conditioned medium. In MLTRZ cells, LPS did not affect the activity of the LTR. Activated macrophages thus secrete suppressor(s) of the MLV-LTR.

Role of macrophage-derived cytokines in the inactivation of the MLV-LTR. Activated macrophages produce various mediators including peptide factors. Previous studies have indicated that certain cytokines such as interferon-γ attenuate activity of the retroviral LTR (12,13). To examine whether macrophage-derived cytokines have an ability to suppress the MLV-LTR, effects of IL-1β, TNF-α and TGF-β1 were tested. Northern blot analysis showed that NR8383 macrophages abundantly expressed these cytokines in response to LPS (Fig. 5A). When added to BAGMAC^{NR} macrophages, TGF-β1 inhibited activity of the LTR (Fig. 5B). Similarly, TNF-α and TGF-β depressed activity of the LTR in MLTRZ mesangial cells (Fig. 5C). In contrast, activity of the SV40 promoter was enhanced by TNF-α in BAGMAC^{NR} cells and by IL-1β in MLTRZ cells. The MLV-LTR is thus selectively inactivated by cytokines elaborated by activated macrophages.

Previous reports have shown that activity of the MLV-LTR may be silenced via methylation (14,15). To examine whether the cytokine-triggered inactivation of the LTR is associated with DNA methylation, BAGMAC^{NR} cells were pre-stimulated by LPS and then treated with a DNA-demethylating agent, 5-azacytidine. X-gal assay and Northern blot analysis showed that the depressed LTR activity in the stimulated, reporter macrophages was not recovered by the treatment with 5-azacytidine (data not shown).

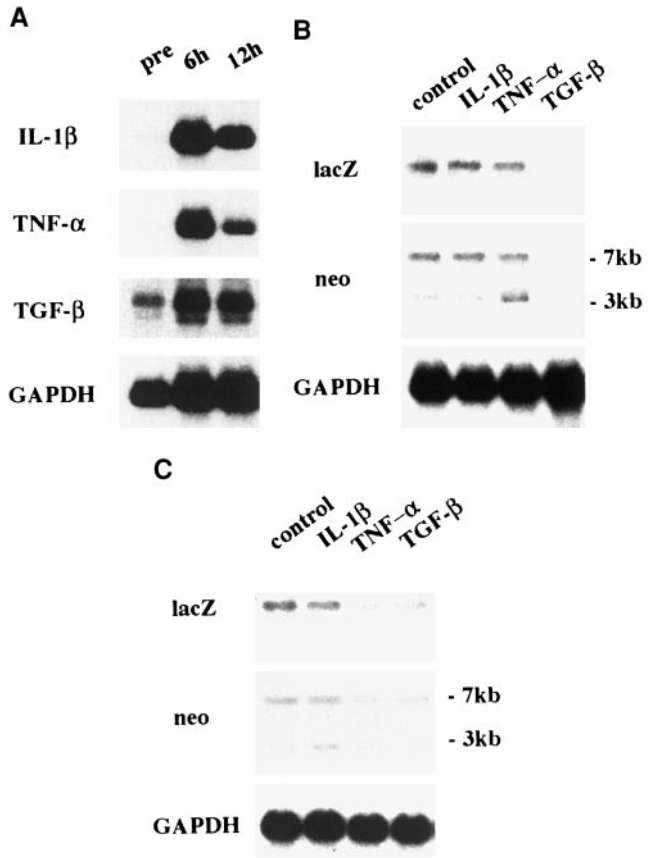

FIG. 5. Role of macrophage-derived cytokines in inactivation of the MLV-LTR. **A.** Expression of cytokines in activated macrophages. NR8383 macrophages were stimulated by LPS for 6 h and 12 h, and expression of IL-1β, tumor necrosis factor-α (TNF-α) and transforming growth factor-β1 (TGF-β1) was examined by Northern blot analysis. **B.** Effect of cytokines on the activity of the MLV-LTR and the SV40 promoter in BAGMAC^{NR} macrophages. BAGMAC^{NR} cells were exposed to IL-1β (10 ng/ml), TNF-α (100 U/ml) or TGF-β1 (20 ng/ml) for 24 h and subjected to Northern blot analysis of *lacZ* and *neo*. **C.** Effect of cytokines on the activity of the MLV-LTR and the SV40 promoter in MLTRZ mesangial cells. MLTRZ cells were exposed to cytokines and subjected to Northern blot analysis.

DISCUSSION

In the cells transduced with the MLV-based retroviral vector, expression of transgenes driven by the LTR was down-regulated by activated, bystander macrophages. Among several cytokines tested, TNF-α and TGF-β were identified as possible, macrophage-derived deactivators of the LTR. Currently, molecular mechanisms involved in the cytokine-mediated suppression of the LTR are not identified, but some possibilities may be postulated. The MLV-LTR contains positive regulatory elements that bind ubiquitous nuclear factors of mammalian cells. Those include; binding sequences for leukemia virus factors and core-binding factors, the NF-1 motif and the glucocorticoid response element (16). The MLV-LTR also contains negative regulatory elements that mediate suppression of the LTR. For example, the stem cell-specific repressor binding site is implicated in the repression of the MLV-LTR in embryonal carcinoma cells and embryonic stem cells (17). Macrophage-derived cytokines may reduce

activity of the LTR via suppression of transcriptional activators or via induction or stabilization of transcriptional repressors.

The retroviral construct used in this report contains the MLV-LTR and the SV40 promoter to drive transgene expression. Previous reports suggested possible interference between 5' and 3' promoters (18,19). For example, an activated 3' SV40 promoter may cause *cis*-acting repression of the 5' LTR. However, the phenomenon observed in this report cannot be explained by this mechanism. As demonstrated in Fig. 3B, the suppression of the MLV-LTR was not always associated with activation of the SV40 promoter. That is, at day 3, the level of the 7 kb mRNA driven by the LTR was markedly suppressed without increase in the level of 3 kb mRNA driven by the SV40 promoter. This result excludes the possibility that promoter interference by the activated 3' promoter was causative of the depressed LTR activity.

The MLV-based retroviral vector has been widely utilized for experimental and human gene therapy. In this regard, the experimental results presented here imply limitations of the MLV-based system, especially for the treatment of infectious and non-infectious inflammation in which various cytokines are involved. In human immunodeficiency virus (HIV) infection, for example, therapeutically-relevant genes must be transferred to and expressed in monocytes/macrophages and lymphocytes. Although the MLV-based vector has been regarded as a useful vehicle for this purpose, the transgene expression may be attenuated *in vivo* via some mechanisms. First, immune cells are generally activated in HIV-infected individuals in response to HIV or opportunistic pathogens (20). Second, immune cells in HIV patients constitutively secrete various cytokines including TNF-α and TGF-β (20). In HIV-infected patients, therefore, LTR-driven transgene expression may be shut-off by immune cell-derived cytokines in an autocrine, paracrine or endocrine fashion. Likewise, in non-infectious inflammation, infiltrating cells and their producing cytokines play crucial roles in the pathogenesis of diseases. After transduction of local resident cells, expression of therapeutically-relevant genes driven by the MLV-LTR may be down-regulated by co-localized, activated macrophages.

In summary, the present report proposes a novel mechanism by which retrovirus-mediated transgene expression may be attenuated without cell death, deletion of transgenes or methylation of retroviral sequences. The present findings call attention to the use of MLV-based retroviral vectors for gene therapy for certain diseases. After transduction of local resident cells, LTR-driven transgene expression may be attenuated by cytokines elaborated from activated, bystander macrophages. This potential problem should be considered for gene therapy for inflammatory diseases in which activated macrophages are commonly involved.

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