Selective gene transfer to T lymphocytes using coreceptor-specific [MLV(HIV)] pseudotype vectors in a transgenic mouse model

Silke Schüle a, Stefanie Steidl a, Sylvia Panitz a, Cheick Coulibaly b, Ulrich Kalinke c, Klaus Cichutek a, Matthias Schweizer a,*

a Division of Medical Biotechnology, Paul-Ehrlich-Institut, Paul-Ehrlich-Straße 51-59, D-63225 Langen, Germany
b Division of Veterinary Medicine, Paul-Ehrlich-Institut, Paul-Ehrlich-Straße 51-59, D-63225 Langen, Germany
c Division of Immunology, Paul-Ehrlich-Institut, Paul-Ehrlich-Straße 51-59, D-63225 Langen, Germany

Received 20 February 2006; returned to author for revision 9 March 2006; accepted 23 March 2006
Available online 2 May 2006

Abstract

The coreceptor usage of HIV-1 envelope proteins (Env) is mainly dependent on a defined variable region within the V3-loop of Env. Thus, retroviral vectors derived from murine leukemia virus (MLV), which have been pseudotyped with HIV-1 envelope proteins holding different V3-loops, enable selective gene delivery into either CXCR4 or CCR5 positive cultured cells. Here, we tested the distribution of CD4/CCR5-tropic [MLV(HIV)]-pseudotype vectors in transgenic mice expressing CD4 and either CXCR4 or CCR5 of human origin. The specificity of gene transfer was analyzed by ex vivo transduction of spleen cells as well as after i.v. or i.p. injection of transgenic mice. Expression of the transferred marker gene EGFP and vector sequences could be detected exclusively in lymphocytes expressing (hu)CD4 and (hu)CCR5, whereas MLV vectors pseudotyped with the VSV-G envelope glycoprotein mediated gene transfer in mice of all genotypes investigated. These data demonstrated that cell-specific gene delivery via [MLV(HIV)]-pseudotyped vectors, as previously shown for cultured cells, is also achievable in vivo.

Keywords: HIV coreceptor usage; Retroviral pseudotype vectors; In vivo distribution

Introduction

Cell type-specific in vivo gene delivery is one favorable goal in gene therapy. However, basic concerns are the undesirable transduction of many, not disease-related cell types and the fear of germ-line transmission following in vivo administration of viral vectors (Connolly, 2002; Smith, 2003), in particular if vectors are used that can integrate into the genome. Accordingly, up to now, mainly non-integrating vectors such as adenoviral vectors have been used for in vivo gene delivery in clinical trials, whereas in vivo application of retroviral vectors will require efficient targeting of specific cell populations.

The host range of retroviruses is mainly determined by the surface (SU) domain of the envelope (Env) glycoprotein, which mediates binding to the surface receptor (Bartosch and Cosset, 2004; Haynes et al., 2003). Accordingly, besides others, various strategies to target retroviral vectors were based on modification of the viral Env protein. For example, retroviral gene transfer into specific cell types has been attempted by inserting antibody fragments or other targeting ligands into the ecotropic Env glycoprotein of Moloney murine leukemia virus (MoMLV), so far with only limited success (Schnierle et al., 1996; Benedict et al., 1999; Gollan and Green, 2002; Bartosch and Cosset, 2004). Providing retroviral vector particles with adapter antibodies resulted in significant reduction of vector titers (Kasahara et al., 1994; Valsesia-Wittmann et al., 1994; Haynes et al., 2003). As a general rule, modified Env proteins can show specific binding properties; however, they often suffer from low fusion activity, which in turn, leads to inefficient transduction of target cells (Zhao et al., 1999). Other targeting
approaches included linking a blocking domain to the MoMLV Env protein. This prevents infection, unless the blocking domains are cleaved off by proteases. In one such example, matrix-metalloproteases that are predominantly secreted by tumor cells cleaved off a blocking domain, thus enabling selective targeting of tumor cells (Chowdhury et al., 2004; Hartl et al., 2005). Finally, retroviruses are readily amenable to replacement of their Env proteins by those of other viruses, whereby the cell specificity is determined by the foreign envelope. Such so-called retroviral pseudotype vectors have been generated using for example the Env glycoproteins of human hepatitis B virus, Sendai virus, Gibbon ape leukemia virus, or vesicular stomatitis virus t (Sung and Lai, 2002; Spiegel et al., 1998; Wilson et al., 1998; Burns et al., 1993). In most cases, specific tropism of modified vectors has been satisfactorily shown in cell culture experiments only, whereas reports on efficient in vivo targeting are rare (Jiang and Dornburg, 1999; Martin et al., 2002; Morizono et al., 2005).

In line with the targeting approach to use heterologous Env proteins, we and others described that MLV particles can be pseudotyped with a C-terminally truncated variant of a (hu)CD4/(hu)CXCR4-tropic HIV-1 Env glycoprotein (Schnierle et al., 1997; Mammano et al., 1997; Stitz et al., 2000). Thus, the Env glycoprotein conferred the natural tropism of HIV-1 to the [MLV(HIV)] vector particle, which was then shown to specifically transduce human CD4/CXCR4-positive cells. To enable usage of the CCR5 coreceptor as well, the V3-loop of the truncated HIV-1 Env protein was replaced by that of a CCR5-tropic HIV-1 strain to generate a CD4/CCR5-specific [MLV(HIV)] vector. The coreceptor specificity of the resulting CCR5- or CXCR4-tropic pseudotype vector has been verified by in vitro transduction of cultured cell lines expressing CD4 and one of the aforementioned coreceptors (Stitz et al., 1998). Coreceptor switch could also be demonstrated by analogous replacement of the V3-loop of the Env protein of a simian immunodeficiency virus used for pseudotyping of MLV vectors (Steidl et al., 2002). These developments might pave the way for gene therapy protocols focusing on targeting T lymphocytes which are involved not only in inherited diseases such as adenosine deaminase deficiency (ADA) and severe combined immunodeficiency disease (SCID), but also in acquired diseases like acquired immune deficiency syndrome (AIDS) or cancer, e.g., cutaneous T cell lymphomas (CTCLs). For example, Thaler et al. (2003) successfully used [MLV(HIV)]-pseudotype vector particles to transfer a suicide gene for specific transduction of a human T cell lymphoma xenograft in a mouse model resulting in reduction of tumor growth.

In the present study, we investigated the tropism of [MLV(HIV)]-pseudotype vectors in transgenic animal models. Vectors were administered i.v. or i.p. into mice harboring cells which expressed various combinations of human CD4 and CXCR4 or CCR5. Our data demonstrated that [MLV(HIV)] vectors allowed a strictly cell type-specific gene transfer in vivo.

Results

Generation of CCR5- or CXCR4-tropic [MLV(HIV)] pseudotype vectors

[MLV(HIV)]-vectors were produced by use of stable packaging cell lines expressing MLV gag/pol, a packaging signal-positive EGFP-carrying MLV transfer vector, and either a CCR5-tropic or a CXCR4-tropic HIV-1-derived env gene. The stable packaging cell line K52SR20 harbors the CXCR4-tropic env gene of HIV-1 BH10 (Stitz et al., 1998), and the cell clone MBK.15 contains the CCR5-tropic env gene of the same strain modified by replacing the V3-loop by that of CCR5-tropic HIV-1 strain MBK (Steidl et al., 2002). Vectors were harvested from cell culture supernatants, and specificity for CD4 and coreceptors was confirmed by transduction of GHOST-CCR5 and GHOST-CXCR4 cell lines expressing the respective receptors (Cecilia et al., 1998) as described (Steidl et al., 2002). Usually, titers of 5×10⁵ transducing units per ml (TU/ml) or 1×10⁶ TU/ml were obtained for the CXCR4- or the CCR5-tropic vector, respectively, which could be concentrated up to 3-fold by ultracentrifugation.

Transgenic mouse models used for in vivo and ex vivo targeting studies

For investigation of cell-specific gene delivery by [MLV (HIV)]-pseudotype vector particles, we used the mouse strains B6CBAF*C57BL/6 [Tg](hu)CD4/(hu)CCR5 or B6CBAF*C57BL/6 [Tg](hu)CD4/(hu)CXCR4 that were double transgenic for the human CD4 in combination with one of the human coreceptors, CCR5 or CXCR4 (LaCasse et al., 1999). To prove the genotype of each individual mouse, surface expression of transgene products was checked by FACS analysis of peripheral blood mononuclear cells (PBMCs) (Fig. 1A), whereas genotypes were confirmed by PCR analysis (Fig. 1B). Since the transgenic mice used carried heterozygous transgenes, besides double transgenic mice, single transgenics expressing only (hu) CD4, (hu)CXCR4, or (hu)CCR5 were also obtained during breeding.

Receptor-specific ex vivo gene transfer into lymphocytes of transgenic mice

To analyze the ability of CD4/CXCR4- and CD4/CCR5-tropic [MLV(HIV)] vector particles to transduce cells from transgenic mice, we treated spleen lymphocyte suspensions ex vivo. To this end, lymphocytes were isolated by Ficoll gradient from spleens of transgenic mice expressing the combinations of the transgenes as indicated. The splenic lymphocytes typically consisted of about 55% CD3+ T cells and 45% CD19+ B cells as determined by FACS analysis (data not shown). Subsequently, cells were stimulated for 2 days with α-CD3 and α-CD28 antibodies, and successful activation of lymphocytes was monitored by analysis of CD69 and CD86 upregulation (data...
Stimulated lymphocytes were then transduced with CCR5- or CXCR4-tropic [MLV(HIV)] vectors at a multiplicity of infection (MOI) of 0.2–0.4. Six days after transduction, lymphocytes were analyzed by FACS for expression of the marker gene EGFP and the human HIV receptors (Fig. 2). As expected, only cells positive for both (hu)CD4 and (hu)CCR5 could be transduced by the CD4/CCR5-tropic [MLV(HIV)] vectors (Fig. 2A, upper panel), and only cells positive for (hu)CD4 and (hu)CXCR4 could be transduced by the CD4/CXCR4-tropic vectors (Fig. 2A, lower panel). The occurrence of EGFP-expressing CCR5-negative cells after transduction with the CD4/CCR5-tropic vector is probably due to downregulation of CCR5 during lymphocyte cultivation after transduction as described previously (Mengozzi et al., 2001; Brandt et al., 2002) rather than to transduction of CCR5-negative cells.

In three independent experiments, transduction rates were about 10%. In contrast, gene transfer was not detectable in lymphocytes expressing in addition to (hu)CD4 the inappropriate coreceptor, or in lymphocytes expressing only one of the transgenic receptors (Fig. 2B). In summary, we demonstrated specific coreceptor usage of CD4/CCR5- and CD4/CXCR4-tropic [MLV(HIV)] vector particles in primary murine lymphocytes in ex vivo experiments.

Receptor specificity of CD4/CCR5-tropic [MLV(HIV)] vectors in vivo

Transgenic mice expressing (hu)CXCR4 showed a disturbed CD4 T cell homeostasis (Sawada et al., 1998). Furthermore, usage of the murine CXCR4 by CXCR4-tropic HIV Env has been described (Bieniasz et al., 1997; Mitnacht-Kraus and Schnierle, 2002). Therefore, we decided to omit analysis of CXCR4-tropic vector in vivo and focussed on analysis of CCR5-tropic vectors.

Since MLV-derived vectors can transduce only dividing cells, lymphocytes were activated in vivo 1 day prior to vector application by i.p. administration of 100 μg keyhole limpet hemocyanin (KLH). Twenty-four hours later, FACS analysis of peripheral CD4+ cells revealed an increased percentage of CD86-positive T cells of about 16% in comparison to about 6% in naïve mice. In the peritoneal cavity of non-treated mice, about 40% of the lymphocytes were CD86-positive a priori, but this proportion was not increased after KLH stimulation (data not shown).

At first, gene transfer was investigated after i.v. administration of vectors using non-transgenic mice, (hu)CD4, (hu)CCR5, or (hu)CXCR4 single transgenic mice, or (hu)CD4/(hu)CXCR4 or (hu)CD4/(hu)CCR5 double transgenic mice (2 animals per...
A total of $1 \times 10^6$ infectious CD4/CCR5-tropic [MLV(HIV)] vector particles were applied to each mouse by three injections at 24, 32, and 48 h after KLH treatment. Five days later, the animals were sacrificed and the gene transfer was analyzed by fluorescent microscopy and FACS analysis of EGFP expression in lymphocytes from peripheral blood, spleen, or peritoneal cavity, as well as by PCR analysis of vector sequences in spleen, liver, thymus, and bone marrow. Gene transfer was demonstrated exclusively in (hu)CD4/(hu)CCR5 double-transgenic mice by detection of sporadic EGFP-expressing splenic lymphocytes by fluorescent microscopy (data not shown). Vector-specific DNA was also detected in the spleen and liver of (hu)CD4/(hu)CCR5 double-transgenic mice (Fig. 5B), but not in the spleen and liver of other animals. Peripheral blood lymphocytes did not show EGFP expression. Therefore, although low transduction frequencies were observed, the gene transfer detected exclusively in animals of the respective genotype confirmed the specificity of the CD4/CCR5-tropic [MLV(HIV)] vector for cells expressing (hu)CD4/(hu)CCR5.

Next, transduction experiments were performed using the i.p. injection route. In each of four independent experiments, two animals per genotype were investigated. A total of $8 \times 10^6$ infectious CCR5-tropic [MLV(HIV)] vector particles were administered to each mouse by repeated i.p. injection 24, 32, 48, and 56 h after KLH treatment, and the animals were analyzed as described above. Again, gene transfer could be detected by fluorescent microscopy (Fig. 3) and by FACS analysis (Fig. 4) exclusively in (hu)CD4/(hu)CCR5 double-transgenic mice, but not in (hu)CD4, (hu)CCR5, or (hu)CXCR4 single transgenic or (hu)CD4/(hu)CXCR4 double transgenic mice. The efficiency of transduction was significantly higher than after i.v. application: in (hu)CD4/(hu)CCR5 transgenic mice, about 0.15% of lymphoid cells from the peritoneal cavity were found to express EGFP (Fig. 4B), which correspond to about 0.3% of all (hu)CD4/(hu)CCR5 positive target cells (Fig. 4C). Remarkably, positive cells were sporadically also found in spleen but not in peripheral blood by fluorescence microscopy (not shown). Within the lymphoid cells from the peritoneal cavity, about 90% of all EGFP-expressing cells were (hu)CD4 and (hu)CCR5 positive in vivo experiments. The presence of about 8% EGFP-positive cells expressing only (hu)CCR5 but not (hu)CD4 may rather be due to loss of (hu)CD4 expression after transduction of double-positive cells than to transduction of (hu)CCR5 positive/(hu)CD4-negative cells, since no EGFP-expressing cells were detected in (hu)CCR5 single transgenic mice (Fig. 4D) indicating the need for receptor and coreceptor for transduction.

By PCR analysis, vector sequences were detected in spleen (Fig. 5A) and, albeit to a lower extent, in thymus and liver of (hu)CD4/(hu)CCR5 double transgenic mice. Again, vector DNA amplification was negative using organs of mice of all other genotypes, even if a second round of PCR was performed. A summary of the PCR results obtained from two independent PCR assays for each mouse and tissue upon i.v. or i.p. administration of the vector is shown in Fig. 5B.

As a positive control, MLV vectors pseudotyped with the glycoprotein of the vesicular stomatitis virus (VSV-G), which is known to mediate receptor-independent transduction of a high number of target cells from different species (Mastromarino et al., 1987; Tatsuo et al., 2000), were administered i.p. as described above for [MLV(HIV)] vectors to (hu)CCR5/(hu)CD4-, (hu)CD4/(hu)CXCR4- transgenic, and wild type mice (4 animals per group). EGFP expression was detected in lymphocytes from spleen and peritoneal cavity (Fig. 3) of all mice investigated, indicating that transduction worked independently of (hu)CD4 and coreceptor expression. Furthermore, vector sequences were detected by PCR in cells isolated from spleen, bone marrow (Figs. 5A and B), liver, and thymus (Fig. 5B), from mice of each genotype.
all genotypes, confirming that all mouse strains tested were in principle susceptible for transduction with MLV-derived vectors. Taken together, these data demonstrate that [MLV(HIV)] vectors have the ability to mediate coreceptor-specific gene transfer to CD4 positive target cells in vivo, depending on the particular envelope used for pseudotyping. Gene transfer was more efficient when the vector particles were administered i.p. than i.v.

Discussion

The development of targeted vectors is crucial for many in vivo approaches in human gene therapy. The ultimate goal
would be the systemic delivery of gene transfer vectors that may include the possibility for repeated administration. With respect to safety issues related to ectopic transgene expression and chromosomal vector integration, it is essential that these vectors are highly specific for transduction of selected target cell types. In this regard, we and others previously described pseudotyping of MoMLV particles with the Env glycoprotein of HIV-1, which then displayed in vitro the natural tropism of HIV-1 to CD4-positive human cells (Schnierle et al., 1997; Mammano et al., 1997; Stitz et al., 2000). These vectors are probably the most efficient cell type-specific vectors generated by pseudotyping (Haynes et al., 2003). Thaler et al. (2003) reported selective transduction of human CD4 cancer cells by such vectors in a murine xenograft model. The present report demonstrates for the first time the tropism of [MLV(HIV-CCR5)] vectors upon i.v. and i.p. injection in transgenic mice. In particular, in vivo administration of vectors pseudotyped with HIV-1 envelopes specific for CD4/CCR5 proved the need for the (hu)CCR5 coreceptor in addition to (hu)CD4 for transduction.

It has previously been reported that the murine variant of CXCR4 is a functional coreceptor for CXCR4-tropic [MLV (HIV)] vectors after transfection of mammalian cells with (hu) CD4 and (mu)CXCR4 (Bieniasz et al., 1997). Also endogenous CXCR4 present in the murine cell line TA3 expressing human (hu)CD4 is able to mediate HIV-1 infection (Mitnacht-Kraus and Schnierle, 2002). In contrast, our data on the ex vivo transduction of primary lymphocytes isolated from spleens of transgenic mice suggested the requirement for the respective human coreceptors in addition to the human CD4 receptor (Fig. 2B). However, in accordance to the data of Schabath et al. (1999), we found that only 11% of murine splenocytes were positive for murine CXCR4 in non-transgenic mice as well as in all transgenic mouse strains investigated. Accordingly, since about 30% of the splenocytes were (hu)CD4-positive, only about 3% of splenocytes from (hu)CD4-transgenic mice were found double-positive for both, human CD4 and murine CXCR4. Moreover, the expression level of murine CXCR4 in single transgenic murine splenocytes was significantly lower.
than on the (hu)CD4-expressing TA3 cells as determined by FACS analysis (data not shown). Thus, absence of ex vivo transduction of splenocytes from (hu)CD4 single transgenic mice via the endogenous murine CXCR4 coreceptor may be due to low murine CXCR4 expression as already suggested by Sawada et al. (1998).

Further on, Sawada et al. (1998) reported that expression of (hu)CXCR4 in lymphocytes of transgenic mice resulted in a disturbed CD4 T cell homeostasis in vivo, whereas transgenic mice expressing (hu)CCR5 did not reveal any abnormalities of the hematopoietic system (Keppler et al., 2002). This was confirmed by our data, since only about 3% of the lymphocytes from the peripheral blood but about 30% of the spleen lymphocytes of CXCR4-transgenic mice were found to be (hu)CD4-positive, whereas in (hu)CCR5-transgenic mice about 30% of the lymphocytes were CD4-positive both in the peripheral blood as well as in the spleen (data not shown). Therefore, to avoid confusing results due to possible usage of murine CXCR4 as coreceptor, as well as to the disturbed CD4 homeostasis in (hu)CXCR4 transgenic mice, we focused our in vivo analysis on properties of the CCR5-tropic [MLV(HIV)] vector.

The clear-cut result on the in vivo transduction capacity of this vector exclusively for lymphocytes of mice transgenic for both (hu)CD4 and (hu)CCR5 was further confirmed by the analysis of EGFP-expressing cells, that were positive for both
(hu)CD4 and (hu)CCR5. Transduction experiments using VSV-G-pseudotyped MLV vectors proved that also wt and (hu)CD4/
(hu)CXCR4-transgenic mice were permissive for transduction with MLV vectors, thus verifying that the in vivo specificity of
the CCR5-tropic vector observed was indeed due to receptor and coreceptor expression.

The efficiency of transduction of cells from the appropriate mouse strains was comparable with previous results. Ex vivo
transduction efficiencies for lymphocytes were similar for the CCR5-tropic and the CXCR4-tropic vector and was about 10%
when an m.o.i. of 0.2–0.4 was used, which was in the same range as described for human peripheral blood lymphocytes in similar
approaches (Indraccolo et al., 1998; Sitz et al., 1998; Thaler and Schnierle, 2001). In vivo, gene transfer rates were very low
when vectors were administrated i.v. Only sporadic EGFP-expressing cells could be detected in the spleen, and
amplification of vector DNA from other organs was only demonstrated for human peripheral blood lymphocytes in similar
approaches (Indraccolo et al., 1998; Sitz et al., 1998; Thaler and Schnierle, 2001). In vivo, gene transfer rates were very low
when vectors were administrated i.v. Only sporadic EGFP-expressing cells could be detected in the spleen, and
amplification of vector DNA from other organs was only successful in some of the animals. In previous reports on higher
transduction rates after i.p. administration resulted in about 0.15% EGFP-expressing peritoneal lymphocytes detectable by fluorescence microscopy or FACS analysis when 8×10⁶ TU were used. This was in the
same range as already described for a comparable rat model under similar conditions (Keppeler et al., 2002). In that report,
the authors administered i.p. 9×10⁶ infectious units of a replication-competent HIV-1 strain, resulting in 0.15% infected lymphocytes detected by EGFP expression. The lack of vector sequences detectable by PCR in other organs of some of the mice confirmed the predominance of vectors in lymphocytes of the peritoneal cavity. Thus, although the low gene transfer rates of the vectors investigated are far from those necessary for in vivo gene therapy approaches, they reflect the current capacity of such vectors and are sufficient to accomplish the goal of our study which was the in vivo examination of target cell specificity.

The reason for the higher transduction rates after i.p. in comparison to i.v. administration of CD4/CCR5-tropic [MLV
(HIV)] vectors remains unclear. It can be speculated that following i.v. application, the vector particles are rapidly disseminated within the blood circuit and may contact and stick to a variety of blood cells or to the endothelium, thus becoming unavailable for transduction of target cells. Never-
theless, negative selection of transduced lymphocytes induced by the transduction process cannot be excluded, for example
due to modulation of lymphocyte activation and homing. Within the peritoneal cavity, the retention period of vector
particles is expected to be significantly higher due to lower circulation rates. Accordingly, EGFP-expressing lymphocytes
could be detected in the peritoneal cavity, but not in the peripheral blood. Transduced lymphocytes detected after i.p.
injection in the spleen and vector sequences detected in thymus and liver may reflect lymphocyte migration from the peritoneal
cavity (Luettig et al., 2001), or accumulation of peripheral lymphocytes after i.v. application. The higher efficiency of i.p
administration may be attributable to the higher dosage achievable due to the higher volume which could be administered i.p., or the higher activation level of lymphocytes as proven by evaluation of activation markers.

In conclusion, we have shown that the coreceptor-specificity of HIV envelopes determined by the V3-loop of Env previously demonstrated in cell culture experiments is also preserved in vivo. Thus, a coreceptor-specific in vivo gene transfer into CD4-positive cells is possible, emphasizing the suitability of [MLV(HIV)]-pseudotyped vectors as valuable tools for gene therapy approaches targeting T lymphocytes. However, our results underline problems of systemic i.v. application of viral vectors such as the needs for high titer vectors for in vivo gene delivery.

Materials and methods

Cell culture

The human kidney cell line 293T (ATCC CRL-11268), the TELCeB6-derived (Cosset et al., 1995) packaging clones K52SR20 (Stitz et al., 1998) and MBK.15 (Steidl et al., 2002), as well as the human osteosarcoma cell lines GHOST-
CCR5 and GHOST-CXCR4 (Cecilia et al., 1998), kindly provided by Dr. M. Dittmar (DKFZ, Heidelberg, Germany),
were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated FCS (Seromed,
Biochrom KG, Berlin, Germany), 100 μM streptomycin/ penicillin, and 2 mM l-glutamine.

The stable packaging clones K52SR20, harboring the CXCR4-tropic env gene of HIV-1 BH10 (Stitz et al., 1998), and the cell clone MBK.15, harboring the CCR5-tropic env gene of HIV-1 BH10 modified by inserting the V3-loop of MBK (Steidl et al., 2002), were permanently cultured in the presence of 5 μg/ml blasticidin S (ICN; Biochemicals, Logan,
UT), 800 μg/ml G418 (Gibco/Life Technologies, Eggenstein,
Germany), and 4.5 g/l glucose. The CXCR4- or CCR5-carrying
GHOST cells were maintained under antibiotic selection in the presence of 100 μg/ml hygromycin (Gibco/Life Technologies),
500 μg/ml G418 (Gibco/Life Technologies), and 1 μg/ml puromycin (ICN, Biochemicals).

Vector production, concentration, and titration

For [MLV(HIV)] vector production, the packaging clones K52SR20 and MBK.15 were cultured in 10-stack cell factories
(Nunc, Wiesbaden, Germany) until they reached confluence. To increase vector production, the culture medium was removed
16 h before harvesting and exchanged with 500 ml medium
containing 6 mM sodium butyrate (BIOMOL, Hamburg,
Germany). VSV-G pseudotyped MLV-vectors were produced by transient transfection, 1.8×10⁷ cells were seeded on T175
culture flasks 24 h before gene transfer. Cells were transfected with 18 μg of the retroviral transfer vector pMGEFGP-
ΔLNGFR, which codes for EGFP (Clontech, Palo Alto, CA) and truncated low-affinity human nerve growth factor receptor
(ΔLNGFR) which are expressed under the control of a murine
stem cell virus (MSCV) LTR, 18 μg of the packaging construct pHIT60 (Soneoka et al., 1995), and 3.5 μg of the VSV-G envelope construct pMD.G (Naldini et al., 1996) using LipofectAMINE Plus, according to the manufacturer’s instructions (GIBCO/Life Technologies). One day after transfection, the medium was replaced by fresh medium and vector containing supernatant was harvested 24 h after medium exchange.

In general, collected supernatants were filtered through 0.45 μm filters and concentrated by ultracentrifugation (25,000 rpm for 2 h at 4 °C in a Sorval SW28 rotor) in aliquots of 30 ml of cell culture supernatant through 20% sucrose in PBS. Subsequently supernatants were removed and the vector pellets resolved in 200 μl PBS. Concentrated supernatants were stored at −80 °C.

Vector titers were determined by serial dilution of [MLV (HIV)] supernatants on the respective GHOST cell line and of VSV-G pseudotyped vectors on 293T cells. The number of EGFP-expressing cells was detected 4 days after transduction by FACS analysis and titers were determined by calculating the percentage of EGFP-positive cells.

Animal model

B6CBAF* C57BL/6 mice, transgenic for (hu)CD4/(hu) CXCR4 or (hu)CD4/(hu)CCR5 (LaCasse et al., 1999), were obtained from Taconic Farms, Inc. (Germantown, NY) and housed in a pathogen-free facility. The offspring was screened for transgene expression by FACS analysis of blood cells. All mice were 6 to 12 weeks of age, and a minimum of 2 mice per experimental group were used in all experiments. Mice were i.p. treated with 100 μg KLH (Keyhole Limpet Hemocyanin) and the following day vector stock was injected i.v. (200 μl) at 24, 32, and 48 h or i.p. (1 ml) at 24, 32, 48, and 56 h. On day 5 after transduction, blood was taken and mice were sacrificed to collect organs. Peritoneal lavage was performed by flooding the peritoneal cavity with 5 ml PBS. All animal experiments were in accordance with the German Animal License Regulations (Tierschutzgesetz).

Isolation and transduction of primary mouse cells

Spleen mononuclear cells (SMCs) were prepared by passing minced spleen through a nylon cell strainer and cells were purified by Ficoll Histopaque (density 1.083 g/ml) density gradient centrifugation (Sigma, St. Louis, USA). Cells were maintained in standard RPMI 1640 medium (including 10% heat-inactivated FCS, 100 U/ml streptomycin/penicillin, 2 mM L-glutamine) which was additionally supplemented with 1 mM Sodium Pyruvate (Gibco/Life Technologies), 100 μM NEAA (Non Essential Amino Acids, Gibco/Life Technologies), and 50 μM β-Mercaptoethanol (Gibco/Life Technologies). For activation, 3.5 × 10^6 SMCs/ml were cultured for 2 days in anti-CD3 (10 μg/ml PBS) precoated 6-well dish and 100 μl anti-CD28 (0.1 μg/ml) was added. For transduction, 2 × 10^6 SMCs/ml were seeded into 24-well plates, and 300 μl of vector containing supernatant, IL-2 (100 U/ml), and proteamine sulfate (4 μg/ml) were added. Subsequently, cells were centrifuged for 1 h at 300×g and 31 °C, and medium was exchanged after 6 h. Transduced cells were cultivated for additional 6 days before analysis of transgene expression.

Flow cytometry

Whole blood FACS analysis was performed from heparin-treated blood samples using the DAKO Uti-Lyse kit (DAKO Corporation, Carpinteria, USA) according to the manufacturer’s protocol. Lymphocyte samples were incubated for 30 min with monoclonal antibodies α(hu)CCR5-PE, α(hu)CXCR4-PE, α(hu)CD4-PerCP-Cy5, α(mu)CD3-FITC, α(mu)CD69-PE, and α(mu)CD86-PE obtained from BD Bioscience (Heidelberg, Germany) or α(mu)CD19-PE, α(mu)CD8-PE, and α(mu)CD4-PE obtained from DAKO (DAKO Corporation). EGFP expression was analyzed in the green fluorescence channel (FL-1). Samples were analyzed with a FACSscan cytometer (BD Bioscience) using Cellquest software.

PCR of vector sequences from genomic DNA

Genomic DNA from different tissues and cells was isolated using the DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. PCR amplification for human CD4 and CCR5 from transgenic mice was done by standard PCR using primers as follows: (hu)CD4 (forward): 5′ TGG GCC CAC AGA CTC ACA 3′; (reverse) 5′ GCC CTT CAT CCC TGC TCG TAA AAA 3′; (hu)CCR5 (forward): 5′ CTC GCC ATC TCT GAC TGG TTT TTC 3′; (reverse) 5′ CAG CCC TGT GCC TCT TCT CT CAT and (hu)CXCR4 (forward) 5′ GAA ATG GGC TCA GGG GAC TAT G 3′; (reverse) 5′ CCT TGG CCT CTG ACT GTT GTT G 3′. Detection of egfp-sequences was performed by standard PCR using 1 μg of genomic DNA, whereby 1/3 of the first round of PCR amplification was used for a second round of PCR. A 373 bp fragment was amplified, using primers as follows: EGFP (forward): 5′ CGTCCAGGAGCGCACCATTCTTCTT 3′ and (reverse) 5′ ATCGCGCTTCTGTTGGGTTCTT 3′. As internal control, a 390 bp-fragment of the human gene β-actin was amplified. As positive control, the respective plasmid (pMG.egfpΔlngfri) was used.

Acknowledgments

We thank Roland Plesker for help with the animal studies and Christine Stellwagen for excellent animal care assistance. We are grateful to Nina Wolfrum and Christian Herder for constructive discussion and for help with the manuscript. This work was supported by grants #0311713, #01KI9718 and #01KV9919 of the BMBF.

References


Tatsu, H., Okuma, K., Tanaka, K., Ono, N., Minagawa, H., Takade, A., Matsura, Y., Yanagi, Y., 2000. Virus entry is a major determinant of cell tropism of Edmonston and wild-type strains of measles virus as revealed by
vesicular stomatitis virus pseudotypes bearing their envelope proteins. J. Virol. 74, 4139–4145.


