

Efficient and stable MGMT-mediated selection of long-term repopulating stem cells in nonhuman primates

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HSC transplantation using genetically modified autologous cells is a promising therapeutic strategy for various genetic diseases, cancer, and HIV. However, for many of these conditions, the current efficiency of gene transfer to HSCs is not sufficient for clinical use. The ability to increase the percentage of gene-modified cells following transplantation is critical to overcoming this obstacle. In vivo selection with mutant methylguanine methyltransferase (MGMT^{P140K}) has been proposed to overcome low gene transfer efficiency to HSCs. Previous studies have shown efficient in vivo selection in mice and dogs but only transient selection in primates. Here, we report efficient and stable MGMT^{P140K}-mediated multilineage selection in both macaque and baboon nonhuman primate models. Treatment consisting of both O⁶-benzylguanine (O⁶BG) and N,N'-bis(2-chloroethyl)-N-nitroso-urea (BCNU) stably increased the percentage of transgene-expressing cells from a range of initial levels of engrafted genetically modified cells, with the longest follow-up after drug treatment occurring over 2.2 years. Drug treatment was well tolerated, and selection occurred in myeloid, lymphoid, and erythroid cells as well as platelets. Retrovirus integration site analysis before and after drug treatments confirmed the presence of multiple clones. These nonhuman primate studies closely model a clinical setting and should have broad applications for HSC gene therapy targeting human diseases of malignant, genetic, and infectious nature, including HIV.

Introduction

HSC transplantation using allogeneic cells has been a successful treatment modality for a variety of genetic diseases, because the graft stably produces phenotypically normal cells for the lifetime of the individual (1). However, many patients do not have suitable donors for allogeneic transplantation. In addition, allogeneic transplantation is often associated with significant morbidity and mortality from graft-versus-host disease and infectious complications due to immunosuppressive treatment (2). Thus, the infusion of genetically corrected autologous stem cells would circumvent these limitations of allogeneic transplantation (3). Furthermore, new treatment modalities using autologous HSCs harboring a potent anti-HIV transgene could be applied alone or in parallel with highly active antiretroviral treatment for HIV patients with advanced disease to limit disease progression. For all of these gene-modified autologous HSC strategies to be successful, high levels of genetically modified cells are required long term to provide a prolonged clinical benefit.

For treatment of hematopoietic diseases that affect a variety of lineages, such as erythrocytes (hemoglobinopathies and thalassemia) and platelets (PLTs) (Wiskott-Aldrich syndrome), genetic modification at the HSC level is desirable to provide lifelong production of “corrected” hematopoietic lineages. A similar strategy of genetic modification of HSCs is favored to provide lifelong “protected” macrophages and lymphocytes in HIV-infected patients. Successful HSC gene therapy has been unequivocally demonstrated for patients with X-linked SCID (SCID-X1) (4),

adenosine deaminase deficient SCID (5), X-linked chronic granulomatous disease (X-CGD) (6), and adrenoleukodystrophy (ALD) (7). Numerous advances in transduction protocols, including transduction on fibronectin fragment CH-296 (8), the use of multiple cytokines (9, 10), and the use of different vector pseudotypes (11–13), have contributed to gene transfer levels of 20%–30% in clinically relevant nonhuman primates (14). Even with the gene marking levels (defined as the percentage or fraction of genetically modified cells) achieved clinically ranging from approximately 9%–15% in peripheral blood with reduced-intensity busulfan (6) and high-intensity cyclophosphamide and busulfan (7) and levels in the nonhuman primate ranging from 20%–30% in peripheral blood with total body irradiation (TBI) (14), for numerous clinical applications gene marking levels will still likely be below therapeutic thresholds (for review see ref. 15).

Without an intrinsic growth advantage provided by gene-modified cells, such as that observed for SCID-X1, efficient and stable posttransplant in vivo selection will likely be required to increase the percentage of these cells into a therapeutic range for diseases such as thalassemia and other hemoglobinopathies. This approach has several therapeutic applications, such as using anti-HIV transgenes to block infection or limit propagation of HIV or using drug resistance transgenes to limit myelosuppression from chemotherapy during cancer therapy to allow dose intensification. The system that currently meets the criteria for all of these applications is mutant methylguanine methyltransferase (MGMT^{P140K}) (16–20), because of stable multilineage in vivo selection that has been previously described in mice (21) and dogs (22, 23). MGMT^{P140K}-mediated selection does not require chronic administration of pharmacologic agents but instead can be achieved with limited treatments

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using the wild-type MGMT inhibitor O⁶-benzylguanine (O⁶BG) in combination with either *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea (BCNU) or temozolomide (TMZ). It is important to extend MGMT^{P140K}-mediated in vivo selection studies to nonhuman primates and test durable sustained selection, because of the ability to directly translate transduction procedures (i.e., antibodies for HSC enrichment, growth factor cocktail, and vector pseudotype), similar dosing of drugs used for conditioning, and in vivo selection to clinical applications. In addition, the nonhuman primate model is a powerful model for diseases such as HIV, which can be modeled using SHIV (24). With regard to safety, studies in the nonhuman primate are critical to assess the long-term implications of cytotoxic drug treatment (BCNU and TMZ), for which the method of action is DNA damage, before proceeding to clinical applications. This long-term data will be especially important for application to nonmalignant disease, for which drug treatments with O⁶BG and BCNU or TMZ would be new treatment modalities. Given these considerations, successful studies in the nonhuman primate should readily translate to clinical applications.

Here, we tested MGMT^{P140K}-mediated in vivo selection and chemoprotection using gammaretrovirus- and HIV-derived self-inactivating (SIN) lentivirus-based vectors in baboons (*Papio cynocephalus*) and pigtailed macaques (*Macaca nemestrina*). We also extended these studies, using bicistronic and tricistronic vectors that allow for in vivo selection of gene-modified cells that also express anti-HIV transgenes, and also included reduced-intensity conditioning, using fractionated busulfan in the macaque. To accompany these efficacy studies and given the adverse events associated with gammaretrovirus-based vectors in clinical trials for X-linked immunodeficiency (SCID-X1) (25) and clonal expansion in X-CGD (6), we also carried out retrovirus integration site (RIS) mapping, before and after drug treatment, to monitor any early indication of clonal restriction that may lead to leukemic transformation. The combination of these studies, along with long-term analysis following drug treatment, is critical to demonstrate the feasibility, safety and efficacy of MGMT^{P140K}-mediated in vivo selection in an important preclinical model. Our data supports the use and continued development of this strategy for chemoprotection during human cancer therapy or to mediate in vivo selection for genetic or infectious diseases affecting the hematopoietic system.

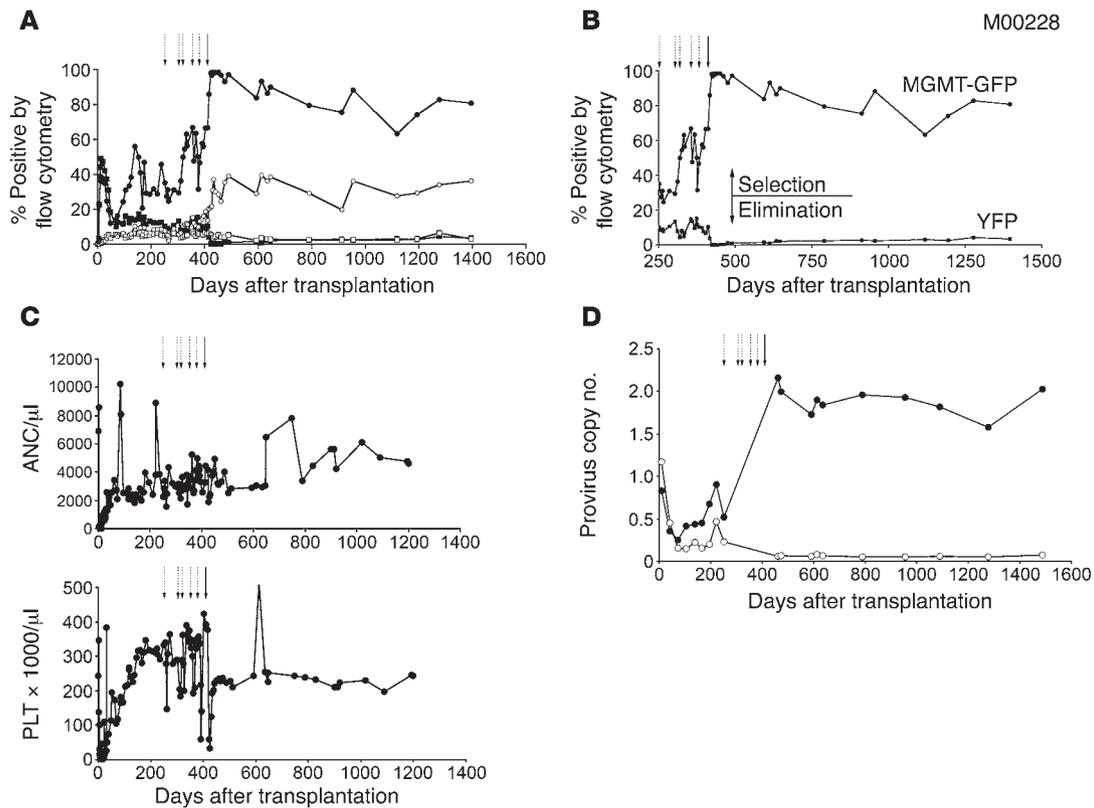
Results

Efficient MGMT^{P140K}-mediated in vivo selection and chemoprotection in baboons and macaques following transplantation with cells genetically modified with gammaretrovirus vectors. HSC gene therapy for a variety of severe hematopoietic genetic, infectious, and malignant diseases will likely require an efficient and robust in vivo selection strategy to attain therapeutic levels of gene marking (defined as the presence of genetically modified cells). Here, we tested the MGMT^{P140K}-mediated in vivo selection in the well-established and clinically relevant baboon and macaque nonhuman primate models. We first evaluated gammaretrovirus vectors expressing MGMT^{P140K} alone or with a fluorescent reporter EGFP and, in some cases, included cells genetically modified with enhanced yellow fluorescent protein (YFP) only as a control arm (26). Drug treatment was initiated after stable hematopoietic recovery and relatively stable gene marking, at least 90 days after transplantation (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI40767DS1). Initially, baboons M01044 and M00228 were given 3 cycles and 1 cycle, respectively,

of TMZ, with a single dose of O⁶BG before TMZ (Supplemental Table 2). This resulted in transient in vivo selection (Figure 1, A and B, and Supplemental Figure 1B, dashed arrows) with no pronounced myelosuppression (Figure 1C). Both monkeys went on to receive TMZ dose escalation, adding a second dose of O⁶BG (O⁶BG-2X) 7–8 hours after TMZ administration to maximize inhibition of wild-type MGMT, further sensitizing unmodified cells (27) (Supplemental Table 2). This drug regimen strategy also resulted in transient in vivo selection (Figure 1, A and B, and Supplemental Figure 1B), with no pronounced myelosuppression (Figure 1C), even with TMZ doses of up to 1,100 mg/m² (M01044) and 1,400 mg/m² (M00228). As an alternative drug regimen for in vivo selection, we next tested the combination of O⁶BG-2X and BCNU, which we had previously shown to be very effective in the dog model, to facilitate MGMT^{P140K}-mediated in vivo selection in primates (23, 28). A drug treatment consisting of O⁶BG-2X and BCNU resulted in substantial in vivo selection of MGMT^{P140K} gene-modified cells (Figure 1, A and B, and Supplemental Figure 1, see selection) and nearly a complete elimination of the YFP-only (unprotected) gene-modified cells (Figure 1, A and B, Supplemental Figure 1, see elimination, and Supplemental Figure 2). Gene marking was monitored in baboons M01044 and M00228 by flow cytometry of the GFP- and YFP-positive cells (representative gene marking plot and flow cytometry in Supplemental Figure 3 of another baboon, M01277). To verify in vivo selection of the MGMT^{P140K}-GFP cells and elimination of the YFP-only cells using a DNA-based assay, we tracked cells using GFP-specific and YFP-specific real-time PCR (RT-PCR) primers and probes. As depicted in Figure 1D, following O⁶BG-2X and BCNU treatment, the trend of MGMT^{P140K}-GFP in vivo selection and YFP-only elimination determined using RT-PCR followed the same trends determined using flow cytometry for the same samples in Figure 1, A and B.

We then wanted to extend the MGMT^{P140K}-mediated in vivo selection studies to the pigtail macaque (*M. nemestrina*), as this is a pertinent preclinical model for anti-HIV strategies (29). After stable hematopoietic engraftment, we observed gene marking, with a provirus copy number of approximately 0.04, determined using RT-PCR, in total wbc of animal M02426. We then dose-escalated O⁶BG-2X and BCNU treatments (Supplemental Table 2). Following 4 cycles of O⁶BG-2X and BCNU treatments, gene marking stabilized at a provirus copy number of approximately 0.55 (Supplemental Figure 4A). The increase in gene marking was verified with retrovirus-specific PCR of individual CFUs following the last cycle of drug treatment in which 62% of individual CFUs contained provirus (data not shown). Following the final dose of O⁶BG-2X and BCNU, there was no pronounced neutropenia, while a control macaque with no MGMT^{P140K} gene marking showed pronounced neutropenia at a lower dose of BCNU (Supplemental Figure 4B).

Gene-modified cells maintain multilineage hematopoietic repopulation potential following MGMT^{P140K}-mediated in vivo selection. Many important hematopoietic and infectious diseases require stable multilineage in vivo selection, because more than one hematopoietic lineage is affected. To achieve stable multilineage selection, the strategy must select HSCs/early progenitors. To establish multilineage selection, we evaluated the level of gene marking in multiple hematopoietic lineages (CD13⁺ granulocytes, CD13⁺ monocytes, CD20⁺ lymphocytes, and CD34⁺ BM cells) before and after MGMT^{P140K}-mediated in vivo selection. Granulocyte and lymphocyte selection was clearly demonstrated in overall hematopoietic populations, based on flow cytometric analysis of MGMT^{P140K}-GFP⁺ and YFP⁺

**Figure 1**

Efficient $MGMT^{P140K}$ -mediated in vivo selection and chemoprotection in the baboon (M00228). (A) Gene marking in $MGMT^{P140K}$ -GFP granulocytes (closed circles), $MGMT^{P140K}$ -GFP lymphocytes (open circles), YFP granulocytes (closed squares), and YFP lymphocytes (open squares), before and after in vivo selection with either O^6 BG and TMZ or BCNU. (B) A truncated representation of the time following in vivo selection with O^6 BG and TMZ or BCNU. Granulocytes are the only subset represented for clarity of selection ($MGMT^{P140K}$ -GFP [$MGMT$ -GFP]) and elimination (YFP). (C) Graphs are the corresponding neutrophil and PLT counts of the same baboon during the drug treatment cycles. (D) Provirus copy number data of $MGMT^{P140K}$ -GFP peripheral blood cells (closed circles) and YFP peripheral blood cells (open circles) of the flow cytometry data represented in A and B. (A–D) Dashed arrows represent in vivo selection with either O^6 BG and TMZ, and solid arrows represent in vivo selection with BCNU.

forward- and side-scatter cell gates (Figure 1, A and B, and Supplemental Figures 1, 2, and 5). Further analysis of more distinct hematopoietic subsets showed the same trend as the overall population, with selection of all the $MGMT^{P140K}$ -GFP⁺ subsets and loss of all unprotected YFP⁺ subsets (Figure 2). The increase in multiple hematopoietic lineages was very stable, as subset analyses were carried out more than 1 year (379 days) after the last drug treatment (O^6 BG-2X and BCNU).

Efficient $MGMT^{P140K}$ -mediated in vivo selection and chemoprotection in macaques following transplantation with cells genetically modified with HIV-derived lentivirus vectors. Multiple retrovirus vectors have been used for stable gene transfer, including gammaretrovirus, HIV- and SIV-derived lentivirus, and foamy virus vectors (30). A variety of factors contribute to the choice of one vector system over the others, but for clinical applications, the RIS profile may be the most important with regard to safety. Both in vitro (31, 32) and in vivo (33–35) RIS profile analyses suggest that lentivirus and foamy virus vectors have an improved safety profile, relative to gammaretrovirus vectors, based on integration in and around promoter regions, specifically those promoters driving proto-oncogene expression (33, 35). To this end, we initiated studies of $MGMT^{P140K}$ -mediated in vivo selection using HIV-derived lentivirus backbones in pigtailed macaques.

Hematopoietic recovery and long-term gene marking prior to $MGMT^{P140K}$ -mediated in vivo selection has been previously described for monkeys J02043, J02370, and T04228 (14). Monkey M05189, which received cells transduced with a tricistronic lentivirus vector containing an anti-HIV transgene (C46), $MGMT^{P140K}$, and GFP, recovered complete blood counts, with normal engraftment kinetics (14) reaching an absolute neutrophil count (ANC) of more than 500 neutrophils per μ l by day 17 (24). Gene marking stabilized in granulocytes and lymphocytes at 5.8% and 4.5%, respectively, by day 249 after transplantation and prior to initiating drug treatment on day 253 (Figure 3). Following each drug treatment, multilineage increases in gene marking were observed in monkeys with $MGMT^{P140K}$ and GFP (Figure 4) and a monkey with an anti-HIV transgene (C46) included in the expression cassette (Figure 3). The increase in gene marking was stable in monkey J02370 upon a follow-up of more than 14 months after the last drug treatment, which resulted in an increase in the gene marking level before drug treatment of 11.3% in granulocytes and 15.3% in lymphocytes to a gene marking level after drug treatment (2 cycles; Supplemental Table 2) of 76.9% in granulocytes and 49.0% in lymphocytes (Figure 4). Furthermore, stable increases in gene marking were also observed in rbc and PLTs, with a gene marking level before drug treatment of 5.6% and 6.7% (14), respectively, and a gene marking level after drug treatment of 15.2%

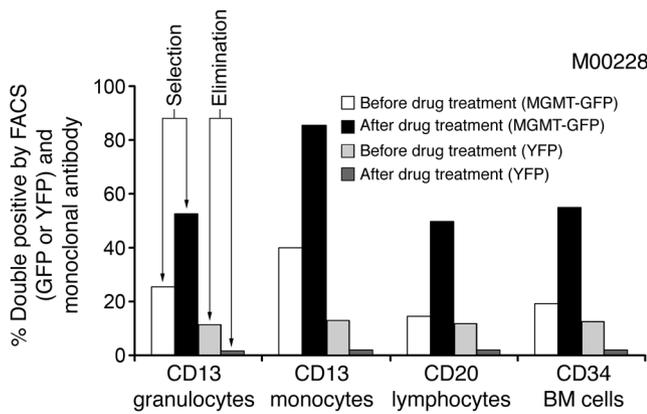


Figure 2 Multilineage MGMT^{P140K}-mediated in vivo selection. Bar graph representation of flow cytometry data from baboon M00228 gated on peripheral blood cells positive for MGMT-GFP or YFP and a monoclonal antibody for the indicated subset. White bars represent MGMT-GFP cells before drug treatment, and black bars represent MGMT-GFP cells after drug treatment (selection). Light gray bars represent YFP cells before drug treatment, and gray bars represent YFP cells after drug treatment (elimination).

and 64.0% (Figure 5), respectively. The EGFP signal is somewhat reduced in rbc, so the gene marking levels reported here are likely an underestimation. Finally, efficient MGMT^{P140K}-mediated in vivo selection was achieved using an HIV-derived lentivirus backbone, with an internal promoter of either elongation factor 1 α (EF1 α) (J02043; data not shown) or spleen-focus forming virus (SFFV) (Figures 3 and 4) expressing MGMT^{P140K}, confirming previous in vitro selection data using similar HIV-derived lentivirus constructs and macaque CD34-selected cells (data not shown).

Efficient MGMT^{P140K}-mediated in vivo selection in macaques following reduced-intensity busulfan conditioning. In the studies described above, we demonstrated efficient and stable MGMT^{P140K}-mediated in vivo selection and chemoprotection following TBI conditioning and transplantation. Since most of the current clinical gene therapy protocols for genetic diseases use a busulfan-based conditioning regimen, we tested targeted busulfan for pretransplant conditioning to provide sufficient myelosuppression and to facilitate engraftment of chemoprotected HSCs, while minimizing extra-hematopoietic toxicity.

Based on previous results testing busulfan conditioning in control monkeys (Supplemental Table 3), we proceeded with a busulfan dose of 4 mg/kg on 2 consecutive days. This dose was well tolerated in the control monkey and resulted in significant neutropenia, indicating efficacy. We hypothesized that this level of myelosuppression would be sufficient to allow for engraftment of gene-modified cells. We also hypothesized that the length of neutropenia observed would be shortened as a result of the gene-modified cell infusion compared with the control animal that did not receive cells. Busulfan blood levels and clearance (Supplemental Methods and Supplemental Table 3) were similar to data previously reported in humans (36).

Following conditioning with busulfan and infusion of gene-modified cells (approximately 1.7×10^7 CD34-selected cells/kg), there was moderate cytopenia, with an ANC of less than 500 neutrophils per μ l for 7 days, and thrombocytopenia, with a nadir of 18,000 PLTs per μ l. Following stable hematopoietic recovery, we observed stable

gene marking, determined using RT-PCR, of 0.04 provirus copies in peripheral blood mononuclear cells that, following a single cycle of O⁶BG-2X and BCNU treatment (Supplemental Table 2, see K05079), rose to 0.16 (Figure 6) in animal K05079. Currently, gene marking has been stable for more than 9 months following drug treatment. Using this reduced-intensity conditioning approach, a high dose of gene-modified cells per kilogram for infusion (37), and posttransplant in vivo selection, we were able to achieve engraftment and selection of MGMT^{P140K} gene-modified cells.

Polyclonal hematopoietic repopulation before and after in vivo selection. In the context of gene-modified in vivo selection following transplantation, either conferred intrinsically by a transgene (i.e., X-SCID) or conditionally during the presence of a biologic molecule or drug (i.e., MGMT^{P140K}), it is important to assess the clonality of hematopoiesis, because of the potential to restrict the clonal repertoire as a result of in vivo selection. It is preferable to have multiple clones contributing to hematopoiesis following standard or gene-modified HSC transplantation to maintain sufficient long-term repopulating cells and clonal repertoire.

We have previously reported 235 unique lentivirus integration sites (RISs) from monkeys J02370 and J02043 prior to drug treatment (33). We have extended the analysis of monkeys J02370 and J02043 and included initial analysis of monkeys M05189, T04228, and K05079 prior to drug treatment and have identified 434 RISs that we believe to be unique (Table 1). Multiple RIS were identified in all monkeys prior to drug treatment; thus, we next wanted to determine whether multiple clones contributed to hematopoiesis following drug treatment, as we have done before in the dog model (38).

In a limited RIS analysis of monkeys, J02370 (RIS analysis on day 883 after the initial transplant and 373 days after the first treatment with O⁶BG-2X and BCNU), J02043 (RIS analysis on day 1,129 after the initial transplant and 837 days after the second treatment with O⁶BG-2X and BCNU), and K05079 (RIS analysis on day 202 after the initial reduced-intensity transplant and 146 days after treatment with O⁶BG-2X and BCNU), we identified 78 sites that we believe to be unique. Of the 78 RISs identified in monkeys J02370, J02043, and K05079, 31 of the RISs were also identified in the initial RIS analysis prior to drug treatment (Table 1). It was clear, even in limited integration analysis following drug treatment, that multiple clones contributed to hematopoiesis in all monkeys analyzed. We wanted to extend this analysis, and considering the

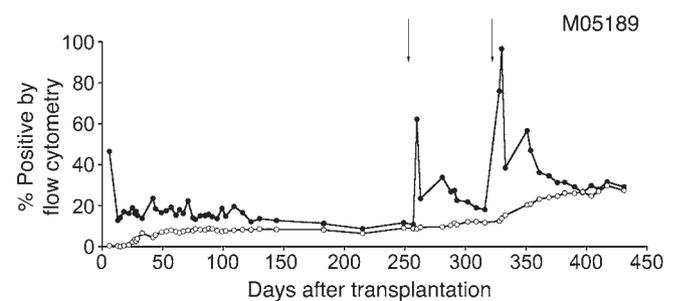


Figure 3 Efficient MGMT^{P140K}-mediated in vivo selection of cells harboring anti-HIV transgenes in the macaque (M05189). Gene marking in granulocytes (closed circles) and lymphocytes (open circles), before and after in vivo selection of MGMT^{P140K}-GFP genetically modified cells in a macaque. Treatment with O⁶BG-2X and BCNU is denoted by solid arrows.

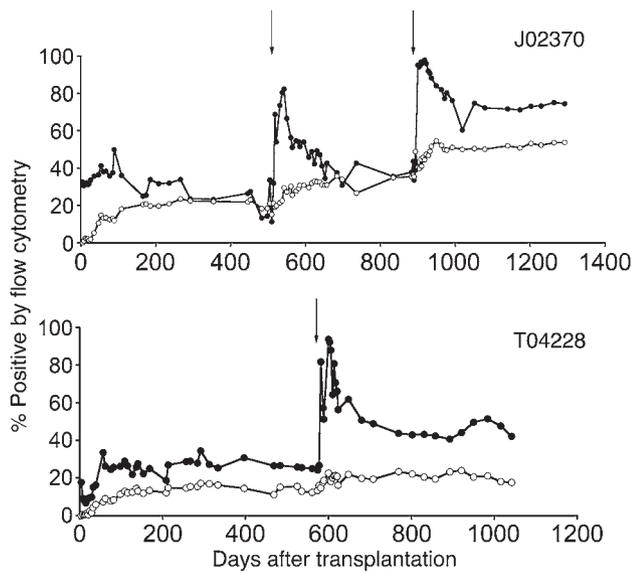


Figure 4
Efficient $MGMT^{P140K}$ -mediated in vivo selection in macaques (J02370 and T04228). Gene marking in granulocytes (closed circles) and lymphocytes (open circles), before and after in vivo selection of $MGMT^{P140K}$ -GFP genetically modified cells in macaques. Treatment with O^6BG-2X and BCNU is denoted by solid arrows.

recurring theme between leukemic transformations in the SCID-X1 clinical trials has been provirus integration near the transcription start site (TSS) of proto-oncogenes, we focused on RIS proximity to proto-oncogene TSSs for safety evaluation. To accomplish this, we carried out integration site analysis both before and after drug treatment (Table 1 and Supplemental Tables 4–6) and mapped the closest proto-oncogene TSS relative to unique RIS and the percentage of RISs within proto-oncogenes. We grouped unique RISs into 3 groups, before drug treatment, after drug treatment, and RISs found both before and after drug treatment (shared), and in all groups, the average distance to the closest proto-oncogene TSS was more than 1 Mb (Supplemental Tables 4–6). The percentages of RISs that mapped within proto-oncogenes from the same 3 groups were 5.2% (before drug treatment), 3.9% (after drug treatment), and 3.2% (shared). This suggests that, following $MGMT^{P140K}$ -mediated in vivo selection, there is not a profound enrichment for RISs in or near proto-oncogene TSSs.

Discussion

Here, we show stable and efficient selection of hematopoietic repopulating cells in a highly relevant nonhuman primate model. We demonstrate stable multilineage selection and chemoprotection in both the baboon (*P. cynocephalus*) and pigtailed macaque (*M. nemestrina*) after O^6BG-2X and BCNU treatment, including selection in PLTs and rbc, with up to 27 months of follow-up after selection. We also demonstrate efficient selection with an HIV-based lentivirus vector, following a reduced-intensity conditioning regimen with single-agent busulfan. Before and after selection, hematopoiesis was polyclonal with both gamma and HIV-derived lentiviral vectors, as determined by linear amplification mediated-PCR (LAM-PCR) in all animals analyzed. Importantly, numerous clones were identified before and after selection, demonstrating that multiple $MGMT^{P140K}$ gene-modified clones can engraft in mon-

keys long-term (14, 26, 33), survive multiple rounds of in vivo selection, and contribute to hematopoiesis following drug treatment. Most importantly, selection was well tolerated in all animals, with acceptable hematopoietic toxicity, transient liver transaminitis, and no pronounced extramedullary toxicity (23). Furthermore, normal hematopoiesis was confirmed before and after drug treatments by histopathology of bone marrow biopsies and bone marrow smears that demonstrated normal cellularity and no indication of malignant progression. These are important studies for the advancement of retrovirus-based gene therapy for clinical applications in which autologous gene-modified cells are appropriate, but in vivo selection strategies will be required to elicit a therapeutic benefit or to enhance chemoprotection during cancer therapy.

For many single-gene hematopoietic diseases or infectious diseases, resolution of the disease will likely require high levels of gene marking (for review see ref. 15). For disorders affecting rbc (i.e., thalassemia and hemoglobinopathies) or PLTs (Wiskott-Aldrich syndrome), high engraftment levels are often required to help alleviate some of the indirect toxicities of the disease, such as iron overload and various organ pathology (39, 40). Also, high engraftment levels are likely required for applications using autologous cells genetically modified with anti-HIV transgenes to provide a significant portion of protected lymphocytes to limit HIV propagation. There are also technical issues that may reduce initial gene marking, resulting from relatively low viral titers and subsequently reduced gene transfer levels, including large complex expression cassettes (i.e., hemoglobinopathies), site-specific targeting vectors, and anti-HIV transgenes that target the HIV-derived lentivirus vector system, thereby directly reducing titers. Therefore, these scenarios will require a robust and stable in vivo selection strategy to increase gene marking to therapeutic thresholds and, ideally, a selection strategy that affects the level of HSCs, so the same system can be used in every situation instead of different systems for each application, depending on the hematopoietic lineage affected. Using both gammaretrovirus and HIV-derived lentivirus vectors, we demonstrate efficient selection of $MGMT^{P140K}$ gene-modified HSCs, based on long-term multilineage selection using the drug combination O^6BG-2X and BCNU, in agreement with our previ-

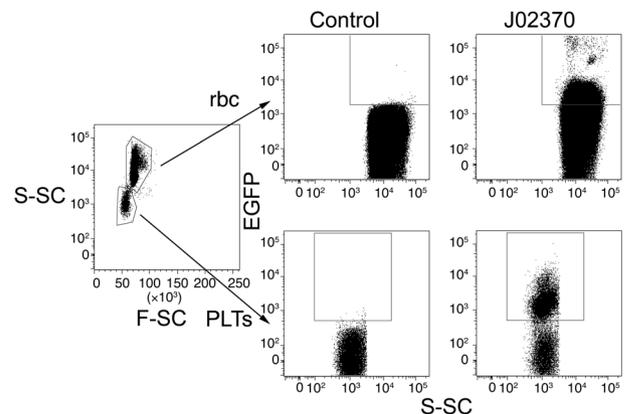


Figure 5
Flow cytometric analysis of transgene-expressing rbc and PLTs following $MGMT$ -mediated in vivo selection. Gating on rbc and PLTs using forward scatter (F-SC) and side scatter (S-SC) and EGFP-expressing rbc (top row) and PLTs (bottom row) plotted by side scatter is shown for a control macaque and macaque J02370.

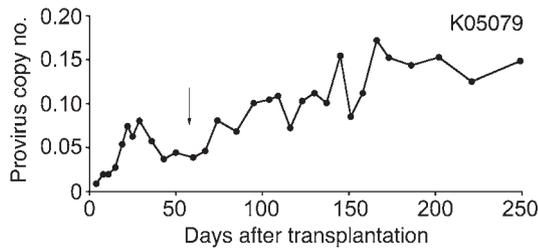


Figure 6 Efficient $MGMT^{P140K}$ -mediated in vivo selection in the macaque following reduced-intensity conditioning (K05079). Gene marking plotted is as provirus copy number from whole wbc, before and after in vivo selection of $MGMT^{P140K}$ -GFP genetically modified cells in a macaque. Treatment with O^6BG -2X and BCNU is denoted by a solid arrow.

ous dog studies (23, 28). However, whereas in dogs, different drug doses are required, especially for BCNU, in monkeys, we used exactly what would be given and has been given to patients, suggesting our approach should readily translate to the clinic.

A recent report by Larochelle et al. suggested that only transient selection could be achieved with $MGMT^{P140K}$ gene-modified cells in the rhesus macaque, with pronounced pulmonary and gastrointestinal (GI) toxicity (41), which contrasts with our studies, which resulted in substantial and stable increases in all hematopoietic lineages analyzed, with acceptable extramedullary toxicity. The major disparities between the studies described by Larochelle et al., our previous reports employing the dog model (22, 23), and the studies described herein include the gene marking reported prior to initiating drug treatment, which, reported by Larochelle et al., was below 3% in the PB of all but one monkey and less than 10% in the remaining monkey, and the drug treatment regimens used for in vivo selection (41).

Low $MGMT^{P140K}$ gene marking levels (<3%) prior to drug treatment make in vivo selection difficult, because few gene-modified cells, contributing to hematopoietic recovery, following a profoundly myelosuppressive dose of cytotoxic drug could result in long durations of cytopenia, which in turn could allow for recovery of unmodified cells that were not killed following drug treatment, thereby diminishing the magnitude of in vivo selection. This is especially true if a suboptimal drug treatment regimen that does not afford an ideal selective advantage for $MGMT^{P140K}$ gene-modified cells relative to unmodified cells is used for in vivo selection. Previously, we have shown that a fractionated regimen of O^6BG and TMZ over 3 days is not as effective as a single bolus of O^6BG and TMZ for in vivo selection in the dog (22). Larochelle et al. exclusively used a 5-day fractionated regimen of O^6BG and TMZ that resulted in only transient in vivo selection. This begs the question: what is the difference between bolus and fractionated regimens using TMZ? The relatively inefficient in vivo selection of $MGMT^{P140K}$ gene-modified cells, using a fractionated drug treatment regimen, is potentially due to the repair kinetics of a variety of DNA adducts following O^6BG and TMZ treatment (42–47). Importantly, in both cases in which we efficiently increased relatively low levels of $MGMT^{P140K}$ gene marking in pigtailed macaques with a provirus copy number of approximately 0.04 (Figure 6), we used O^6BG -2X and BCNU without prior O^6BG and TMZ treatment.

Similar to our previous studies in the dog (22), extending these nonhuman primate studies, we used a single bolus dose of O^6BG and TMZ initially in the baboon and then moved to a fractionated

dose of O^6BG -2X before and after TMZ (27). Initially, we observed transient selection of peripheral blood granulocytes, with no pronounced myelosuppression. This suggested that dosing was suboptimal for HSC selection; however, upon dose escalation of TMZ to levels far above what is dose limiting in humans (48), we continued to see transient granulocyte selection, without pronounced myelosuppression. It should be noted that the lack of myelosuppression may have been misleading, as the $MGMT^{P140K}$ gene marking level at the time of O^6BG and TMZ treatment would not have indicated pronounced myelosuppression based on our previous dog studies (22). The YFP-only cells (unprotected) were essentially unaffected by the O^6BG and TMZ treatments, suggesting that the delivery of TMZ to the 2 baboons may not have been optimal, but with a single dose of O^6BG -2X and BCNU, the YFP-only cells were almost completely eliminated, while the $MGMT^{P140K}$ -GFP cells showed robust and stable selection (26). This data, combined with previous data in the nonhuman primate (41), suggest that $MGMT^{P140K}$ gene-modified cells are effective at preventing myelosuppression following O^6BG -2X and TMZ treatment, but as of yet, an optimum O^6BG and TMZ drug regimen has not been established for in vivo selection as previously demonstrated in the dog (22). Studies are currently ongoing to test new methods for efficient drug delivery using the new IV formulation of TMZ as well as using dacarbazine as an alternative drug that affects cell killing through the same active metabolite, MTIC (for review see ref. 49).

Regardless, unlike in the macaque studies reported by Larochelle et al. (41), both baboons were able to tolerate a single dose of O^6BG -2X and BCNU following multiple rounds of O^6BG and TMZ. Although we have achieved promising results in both baboons and pigtailed macaques using $MGMT^{P140K}$ -mediated in vivo selection, with acceptable toxicity, we believe it is still crucial to minimize the number of drug treatment regimens, especially when applying this drug resistance strategy to increase gene-modified cells to therapeutic levels for genetic or infectious diseases in which drug treatment is not the standard of care. However, this still does not address the disparity in the extramedullary toxicity seen in rhesus macaques (41). A patient population for which there

Table 1 Source of integration sites analyzed

Monkey	RISs before drug treatment	RISs after drug treatment
J02370 ^A	88	26
J02043 ^A	241	36
T04228	70	NA
M05189	28	NA
Total	427	62
Shared	–	30
K05079	7	16 ^B
K05079 granulocytes	NA	11 ^C
K05079 lymphocytes	NA	7 ^D
Total	7	16
Shared	–	11/3 ^E

To our knowledge, all RISs discussed in this table are unique. ^AWe have previously reported 235 unique RISs (33). ^BRISs identified from bulk DNA, lymphocyte-sorted DNA, and granulocyte-sorted DNA. ^CRISs identified from granulocyte-sorted DNA. ^DRISs identified from lymphocyte-sorted DNA. ^EShared RIS found before and after treatment. ^FShared RISs after treatment in sample from bulk DNA, lymphocyte-sorted DNA, and granulocyte-sorted DNA. NA, not analyzed.



is extensive data with regard to the toxicity of O⁶BG, BCNU, and TMZ includes patients with various types of brain tumors (for review see ref. 50). Although combinations of O⁶BG-BCNU and O⁶BG-TMZ have been effective at reducing endogenous MGMT activity in the tumor and at the same time sensitizing the previously refractory tumor, O⁶BG exacerbates the dose-limiting hematopoietic toxicity of both BCNU and TMZ, while the extramedullary toxicity has been acceptable (51, 52). Extramedullary toxicity associated with BCNU is usually pulmonary toxicity caused by progressive fibrosis (for review see ref. 53). Although this is well documented, early onset/acute pulmonary toxicity associated with BCNU is rare and always associated with high-dose BCNU (54, 55). These findings would argue that the pronounced pulmonary and GI toxicity in the study by Larochelle et al. (41) more than likely associated with the heavy pretreatment of the monkeys with TBI and multiple rounds of 5-day fractionated O⁶BG-TMZ prior to O⁶BG-BCNU and/or an unexpectedly high sensitivity of rhesus macaques (*Macaca mulatta*) to BCNU.

Based on these findings, we believe that MGMT^{P140K} strategies to mediate in vivo selection for genetic and infectious diseases affecting the hematopoietic system or chemoprotection during cancer therapy are promising clinical approaches. We demonstrate efficient in vivo selection, beginning with gene marking levels as low as approximately 4%, and based on recent clinical studies (6, 7), it is reasonable to expect to routinely achieve this level of gene marking using established, well-tolerated clinical conditioning regimens (reduced intensity and high intensity or myeloablative). The selection strategies, described here in nonhuman primates, using O⁶BG-2X and BCNU should be readily translatable to the clinic, with respect to drug dosing relative to gene marking, but we also recognize that initial cell dose, conditioning regimen, gene transfer efficiency, and genetic background will all contribute to successful drug resistant clinical gene therapy applications. In particular, when comparing the data presented here, using reduced-intensity conditioning, to previous studies in nonhuman primates (37), the gene marking reported here prior to in vivo selection is generally higher and could be the result of an elevated dose of busulfan used for conditioning (8 mg/kg versus 4–6 mg/kg) and/or a higher cell dose per kilogram. Also, in comparison with recent successful clinical trials for CGD (6) and ALD (7), the cell dose in the monkey that received reduced-intensity conditioning in our study was also higher but generally was within 2–3 fold of the reported values. Although direct comparisons are difficult between the nonhuman primate data and clinical data, gene-modified cell dose and an appropriate conditioning regimen are undoubtedly key components for successful gene therapy applications. With respect to the safety of the studies outlined here, our clonality studies, both before and after in vivo selection, show no obvious indication of progression toward monoclonal hematopoiesis. Although, with the limited RIS analysis, especially in the monkey with reduced-intensity conditioning, and fewer RISs identified, additional monitoring and analysis is critical to assess the long-term safety of the approach, to begin to address what impact the intensity of ablation for conditioning and in vivo selection have on engraftment and clonality of gene-modified cells. As with any experimental treatment approach, safety is a primary concern. To this end, we have included studies with SIN HIV-derived lentivirus vectors that are widely considered to have a safer integration profile relative to gammaretrovirus vectors and reduced-intensity conditioning to minimize transplant-related toxicity. SIN lentiviral vectors

combined with a reduced-intensity conditioning regimen is a potentially promising approach for clinical studies and warrants further investigation. To continue to advance HSC gene therapy, continued research needs to focus on vector development, targeted integration approaches, and regulating transgene expression. For MGMT^{P140K} strategies, additional work to define effective drug regimens that allow for efficient in vivo selection, while minimizing extramedullary toxicity, will be important.

Methods

Animal care and procedures. Healthy juvenile baboons (*P. cynocephalus*) and macaques (*M. nemestrina*) were housed at the University of Washington Regional Primate Research Center under conditions approved by the American Association for the Accreditation of Laboratory Animal Care. Studies were conducted under protocols approved by the Institutional Review Board and Animal Care and Use Committees of the University of Washington.

Transplantation with gammaretrovirus-transduced baboon CD34-enriched cells. Transplantation procedures and protocols have been previously described, and TBI doses for the individual animals are listed in Supplemental Table 1 (26, 56).

Transplantation with gammaretrovirus-transduced macaque CD34-enriched cells. Based on extensive comparisons among different vector backbones, incorporation of the SF91 design using the GRSΔ270 leader results in robust MGMT^{P140K} expression in HSCs (57). This design has a number of modifications in the 5' untranslated leader sequences to remove all AUG codons and all viral coding sequences; thus, the probability of expression of unwanted peptides and the potential for homologous recombination is essentially eliminated (57–59). The backbone for the MGMT^{P140K} gammaretrovirus construct (provided by D.B. Kohn, UCLA, Los Angeles, California, USA) was modified to eliminate all of the *pol* sequences from the 5'-leader region. Four oligonucleotides were designed to amplify the deleted portion of the leader sequence in an asymmetric PCR reaction (SF-A, 5'-TGTCTGCTGCAGCATCGTTCTGTGTTGTCTCTGTCTGACTGTGTTTCTGTATTTGTCTGA-3'; SF-B, 5'-TGGAGAGAGGGACTATTACTTAACCTTGTGTCAGCTAATTTTCAGACAAATACAGAAACAC-3'; SF-C, 5'-AGTAATAGTCCCTCTCTCCAAGCTCACTTACAGCGGCCGCCGCATGGAAGCTTGGG-3'; and SF-D, 5'-CCCAAGCTTCATGCGCGCG-3') (60). The modified leader sequence was cloned into the MND backbone using standard molecular biology techniques, yielding the construct pMND.GRS.P140K.

To create a stable producing line, Phoenix-GALV cells were plated at 1 × 10⁶ cells per well in 6-well tissue culture-treated cluster plates in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Approximately 24 hours after plating, cells were transfected in quadruplicate with the plasmid pMND.GRS.P140K and the blasticidin resistance plasmid pBS.br2, using standard calcium phosphate techniques. The gammaretrovirus plasmid was in either a 20:1 or 100:1 molar excess to pBS.br2, to favor stable gammaretrovirus insertion in blasticidin resistant colonies. Three days after transfection, quadruplicate wells were trypsinized and combined into 3 different 10-cm plates, at increasing cell densities of 1 × 10⁴, 1 × 10⁵, and 1 × 10⁶ cells per plate. The following day, cells began selection with standard media supplemented with 10 ng/ml blasticidin. Media was changed every 4 days, for 12 days in total, with fresh media and blasticidin. After 12 days of selection, individual clones were selected using autoclaved cloning rings. Blasticidin-resistant cells were plated in a 96-well plate and expanded to duplicate 12-well plates as necessary. When cells reached approximately 90% confluency, small scale viral harvests were removed and used to transduce HT1080 cells (duplicate plates were frozen down for cell stocks). Following transduction, HT1080 cells were stained intracellularly with a monoclonal antibody against MGMT (anti-MGMT,



catalog MC-380; Kamiya Company). Clones that stained positive relative to control cells were thawed and tested again on HT1080 cells to determine the highest titer line. The clone PhGALV-MND.GRS.P140K c22 was determined to be the highest titer line and was subsequently frozen down in several aliquots for master cell bank preparation.

Transplantation procedures and protocols have been previously described (26, 56), with the following modifications. First, CD34-selected cells were transduced as previously described (14), with a neat virus containing media (VCM) preload and 2 successive virus exposures with neat VCM at an MOI of 0.5 and 0.5. Second, in preparation for transplantation, the macaque received TBI, 900 cGy, administered from a linear accelerator at 7 cGy/min, as 2 equally divided doses 24 hours apart. After the irradiation, on the second day, approximately 2.5×10^7 PhGALV-MND.GRS.P140K transduced cells/kg were infused through an inserted central vein catheter.

Transplantation with HIV-derived lentivirus-transduced macaque CD34-enriched cells. Transplantation procedures and protocols have been previously described (14), with a few modifications explained below.

The vector developed for anti-HIV strategies (RSC-SC46-ires-MPGw2) was a SIN HIV-derived lentivirus vector based on the RRL system (61) (provided by Didier Trono, École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland) with a backbone containing a central polypurine tract and a woodchuck posttranscriptional regulatory element. The internal promoters were a SFFV promoter expressing the membrane-anchored C-peptide (C46) derived from HIV-1 envelope glycoprotein gp41 (62) linked by an internal ribosome entry site (ires) to MGMT^{P140K} and a phosphoglycerate kinase promoter driving expression of EGFP for monkey M05189 (24). The vector was pseudotyped with VSV-G envelope, produced by transient transfection of 293T cells, and concentrated 100 fold as previously described (63).

The CD34-selected cells were transduced as previously described (26), with 2 successive virus exposures at an MOI of 5 and 4.3. In preparation for transplantation, the macaque received TBI, 900 cGy, administered from a linear accelerator at 7 cGy/min, as 2 equally divided doses 24 hours apart. After the irradiation, on the second day, approximately 2.3×10^7 total cells/kg or approximately 1.1×10^7 RSC-SC46-ires-MPGw2-transduced cells/kg (adjusted based on GFP⁺ gene marking, approximately 49.6%, by flow cytometry of in vitro maintained cells) were infused through an inserted central vein catheter.

Reduced-intensity conditioning and transplantation with HIV-derived lentivirus-transduced macaque CD34-enriched cells. Busulfan was administered to monkey K05079 intravenously over 2 hours (Supplemental Table 3). Gene-modified cells were infused approximately 24 hours after the second dose of busulfan, following our standard lentivirus transduction protocol (14). To avoid any potential immune response to MGMT^{P140K} gene-modified cells, tacrolimus was administered orally twice a day (from day 2 to day 221) following transplantation, and the dose of tacrolimus was adjusted to between 10–15 ng/ml, based on serum concentrations.

Flow cytometric analysis of baboon and macaque hematopoietic cells. Leukocytes, isolated by ammonium chloride red cell lysis from heparinized peripheral blood (PBL) and bone marrow (BML) samples, drawn at multiple time points after transplantation, were analyzed for GFP/YFP-expression on a FACS Vantage or FACSCalibur (Becton Dickinson). These PBLs and BMLs were identified out of at least 20,000 events (1 µg/ml propidium iodide, excluding forward and right-angle light scatter-gated). Flow cytometric data were analyzed by FlowJo version 8.5.2 to version 8.8.6 (Tree Star Inc.) or CELLQuest version 3.1f software (Becton Dickinson), with gating to exclude fewer than 0.1% control cells in the relevant region.

FACS sorting of hematopoietic subsets. Hemolyzed blood or bone marrow was used as starting material for wbc analysis. wbc were counted and labeled with antibodies specific to CD20 (clone 2H7, Becton Dickinson), CD13 (clone L138, Becton Dickinson), and CD34 (clone 563, Becton Dickinson)

in separate tubes. The cells were washed once before analysis on a FACS Aria (Becton Dickinson). Cells were gated using propidium iodide exclusion (as described above); forward and right-angle light scatter was used to distinguish between bulk lymphocytes, granulocytes, and monocytes. Cell lineage gates were further separated by 2-color flow cytometry, using PE-labeled antibodies to the surface markers for CD20⁺ lymphocytes, CD13⁺ monocytes or granulocytes, and CD34⁺ bone marrow cells.

Quantitative PCR (TaqMan). Gene marking was also analyzed by TaqMan 5' nuclease quantitative RT-PCR assay. Sample DNA was analyzed in duplicate with a GFP-specific primer/probe combination (5'-CTGCACCACCGCAA-3'; 5'-GTAGCGGCTGAAGCACTG-3' [Integrated DNA Technologies]; probe, 5'-FAM-CCACCCTGACCTACGGCCTG-TAMRA-3' [Applied Biosystems]), a YFP-specific primer/probe combination (5'-CTGCACCACCGCAA-3'; 5'-GTAGCGGGCGAAGCACT-3' [Integrated DNA Technologies]; probe, 5'-FAM-CCACCTTCGGCTACGGCCTG-TAMRA-3' [Applied Biosystems]), or a lentivirus-specific primer/probe combination (5'-TGAAAGC-GAAAGGAAACCA-3'; 5'-CCGTGCGCGCTTCAG-3' [Integrated DNA Technologies]; probe, 5'-FAM-AGCTCTCTCGACGCAGGACTCGGC-TAMRA-3' [Applied Biosystems]). In a separate reaction, a β -globin-specific primer/probe combination (5'-CCTATCAGAAAGTGGTGGCTGG-3'; 5'-TTGGACAGCAAGAAAGTGAGCTT-3' [Integrated DNA Technologies]; probe, 5'-FAM-TGGCTAATGCCCTGGCCACAAGTA-TAMRA-3' [Applied Biosystems]) was used to adjust for equal loading of genomic DNA per reaction. Standards consisted of dilutions of DNA extracted from cell lines transduced with a single copy of a GFP-, YFP-, or lentivirus-containing retrovirus vector and DNA from the same unmodified cell line. The approximate percentage of genetically modified cells can be calculated by making the assumption that each genetically modified clone we analyzed by PCR has a single copy, meaning that the fraction we report as 0.04 provirus copies can also be reported as a percentage, i.e., 4%. Reactions were run by means of the ABI Master Mix (Applied Biosystems) on the ABI Prism 7500 Sequence Detection System (Applied Biosystems), under the following thermal cycling conditions: 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Drug treatment with O⁶BG and TMZ or BCNU. Drug treatments were always administered under general sedation. We dissolved 50 mg O⁶BG (Sigma-Aldrich) in 30 ml of 40% polyethylene glycol in PBS, and the concentration was adjusted to 1 mg/kg with prewarmed (37°C) PBS. The drug was further diluted in normal saline to a final volume of approximately 150–200 ml and was infused over 15–20 minutes. The dose of O⁶BG for both baboons and macaques was 120 mg/m². After initial drug treatment regimens were tested in baboons M01044 and M00228 (see Supplemental Table 2), a second identical dose of O⁶BG was administered approximately 7–8 hours after the end of TMZ or BCNU administration (27). TMZ capsules (Temodar, Schering-Plough) were crushed and mixed with cranberry juice (pH < 4) and administered via an oral gavage within 5 minutes after completion of the O⁶BG infusion. BCNU (Bristol-Myers Squibb and Sigma-Aldrich) was prepared as previously described (23) and administered 45–60 minutes after the end of the first O⁶BG infusion. Whole blood cell counts and blood chemistry values (including liver and kidney functions) were monitored on the day of drug treatment and twice a week thereafter until values returned to pretreatment levels.

LAM-PCR of RISs. Retroviral integration site analysis by LAM-PCR was performed on primate DNA isolated from PBLs. One hundred nanograms of DNA served as template for LAM-PCR, which was performed as described previously (33) or with a modified approach that was described elsewhere (31, 64).

The analysis of vector proviruses in or near oncogenes was performed using proto-oncogenes, as defined in the Sanger Institute Cancer Gene Census Table (<http://www.sanger.ac.uk/genetics/CGP/Census/>) and the Mouse



Retrovirus Tagged Cancer Gene Database (<http://rtcgd.ncicrf.gov>), as previously described (35), with the following modifications. Oncogenes were localized to the rhesus genome using their human Refseq mRNA sequence, as listed in the xenoRefSeqAli file available from the UCSC genome bioinformatics website (<http://genome.ucsc.edu/cgi-bin/hgTables>). The TSS on the rhesus genome was defined as the first nucleotide where the human Refseq proto-oncogene mRNA sequence aligns to the rhesus genome.

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