Induction of cytotoxic T-lymphocyte responses to enhanced green and yellow fluorescent proteins after myeloablative conditioning

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Lentiviral vectors are increasingly being used for transferring genes into hematopoietic stem cells (HSCs) due to their ability to transduce nondividing cells. Whereas results in vitro and in vivo studies of the nonobese diabetic/severe combined immunodeficiency (NOD/SCID) model have been highly encouraging, studies in large animals have not confirmed the superior transduction of HSCs using lentiviral vectors versus oncoretroviral vectors. In contrast to the stable gene marking we have consistently achieved with oncoretroviral vectors in animals that received myeloablative conditioning, we observed the complete disappearance of genetically modified enhanced green or yellow fluorescent protein–expressing cells in 5 baboons that received transplants of HSCs transduced with lentiviral vectors alone or in combination with oncoretroviral vectors. Immune responses to transgene products have been found to be involved in the disappearance of gene-modified cells after nonmyeloablative conditioning. Thus, we examined whether the disappearance of gene-modified cells after ablative conditioning may be due to an immune response. In 4 of 5 animals, cytotoxic T lymphocytes specific for the transgene protein were readily detected, demonstrating that immune reactions were responsible for the disappearance of the gene-marked cells in the animals. In summary, we report the induction of transgene-specific immune responses after transplantation of lentivirally transduced repopulating cells in a myeloablative setting. (Blood. 2004;103:492-499)

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Introduction

Whether for the treatment of genetic or acquired diseases such as cancer or AIDS, the efficacy of most gene therapy protocols will depend on persistent, high-level expression of transgene-encoded proteins. In many instances these proteins will constitute novel antigens, and thus the induction of immune responses against transgene products is of concern for the long-term success of these therapies. Clinical trials and large animal studies have shown that when cells expressing xenogenic reporter genes or selectable markers such as enhanced green fluorescent protein (EGFP), neomycin resistance, and HyTK are infused into human or large animal recipients without conditioning or after nonmyeloablative conditioning regimens, potent T-cell– and antibody-mediated immune responses capable of clearing the gene-marked cells from the host can develop. In contrast, no immune responses against gene-modified cells have been reported when cells are transplanted after myeloablative conditioning regimens, and recipients generally remain tolerant to the transgene products. The induction of tolerance after myeloablative and nonmyeloablative transplantation has not been carefully studied, but may in part reflect killing or inhibition of peripheral T cells specific for the foreign gene product by the conditioning and the deletion of T cells specific for the foreign gene product generated de novo in the thymus after transplantation.

Recently, a number of research groups have reported the use of lentiviral vectors for the transduction of hematopoietic stem cells. Because lentiviral vectors are able to transduce nondividing cells, they may be superior to oncoretroviral vectors for the transduction of relatively quiescent hematopoietic stem cells (HSCs), especially in relatively short transduction protocols or in the absence of cytokine stimulation. Very high lentiviral transduction of murine stem cells and human nonobese diabetic/severe combined immunodeficient (NOD/SCID) mouse repopulating cells (SRCs) have been reported, with gene transfer levels more than 50%. In contrast, gene-marking frequencies in nonhuman primates have been variable and generally quite low with lentiviral vectors. In a previous study of 6 baboons that received transplants of lentivirus-transduced HSCs after myeloablative conditioning, we achieved persistent long-term gene marking in only 2 animals, and only one showed long-term marking of more than 1% of peripheral blood leukocytes. The complete disappearance of genetically modified cells after myeloablative conditioning was unusual and prompted us to consider whether immune responses could have contributed to this disappearance. Therefore, in the current study we analyzed 5 additional animals that exhibited a complete loss of genetically modified cells after transplantation to determine if an immune response against the transgene played a role in the clearance of the gene-marked cells.

Materials and methods

Lentivirus vectors

The lentiviral transfer vectors RRLsin.cPPT.hPGK.GFP.Wpre and RRLsin.cPPT.hPGK.YFP.Wpre (kindly provided by Dr L. Naldini, San Raffaele Telethon Institute for Gene Therapy, Milan, Italy) are self-inactivating HIV-derived vectors expressing EGFP or its yellow variant (EYFP) from the internal human phosphoglycerate kinase promoter (hPGK), and include a woodchuck hepatitis pre-element and a central polyuridine tract.
Transplantation of NOD/SCID mice with baboon CD34^+ cells

Cells were transplanted into NOD/LtSz-scid/scid (NOD/SCID) mice similarly to a published standard protocol. All mice were bred from breeders purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were handled under sterile conditions and maintained in microisolators. Before transplantation, 6- to 8-week-old mice received TBI with 375 cGy at 20 cGy/min from a linear accelerator source and received transplants by tail-vein injection within 24 hours with 2 × 10^6 CD34-enriched baboon cells after retroviral transduction, after mock transduction or uncultured, as noted.

Flow cytometric analysis of baboon hematopoietic cells

Leukocytes, isolated by ammonium chloride red cell lysis from heparinized peripheral blood and bone marrow samples drawn at multiple time points after transplantation, were analyzed for EGFP and EYFP expression on a FACS Vantage (Becton Dickinson, San Jose, CA). For each sample, at least 500,000 propidium iodide (2 μg/mL)–excluding, forward- and right-angle light scatter–gated events were evaluated. Flow cytometric data were analyzed using CELLQuest v3.3 software (BD Biosciences, San Jose, CA).

Expression of EGFP and EYFP in granulocyte, monocyte, and lymphocyte populations was determined either by gating based on forward- and right-angle light scatter characteristics or on expression of lineage-specific CD markers. Murine antihuman phycoerythrin (PE)–conjugated monoclonal antibodies which have been shown to crossreact with baboon CD markers included anti-CD13 (clone L138), anti-CD20 (clone L27), and matched isotype control (clone X40) from BD Pharmingen, San Diego, CA, and anti-CD3 (clone FN18) from BioSource International, Camarillo, CA. Red cells and platelets from whole blood diluted 1:100 in phosphate-buffered saline (PBS) were delineated by their forward- and right-angle light scatter properties and assessed for EGFP/EYFP expression.

Analysis of EGFP/EYFP expression in a CFU assay

CD34-enriched cells (1000 per 35-mm plate) were cultured at least in triplicate in a double layer agar culture system as previously described. Briefly, isolated cells were cultured in alpha minimal essential medium supplemented with 25% HIFBS, 0.1% bovine serum albumin (BSA; fraction V; Sigma, St Louis, MO), 0.3% (wt/vol) agar (BioWhittaker, Rockland, ME) overlaid on medium with 0.5% agar containing 100 ng/mL SCF, IL-3, IL-6, granulocyte-macrophage–CSF (GM-CSF), and G-CSF, and 4 μg/mL Epo (provided by Amgen). Cultures were incubated at 37°C in 5% CO2 in a humidified incubator. Colonies were enumerated and evaluated for EGFP/EYFP expression at day 14 of culture using an inverted fluorescent microscope. DNA was isolated from colonies and subjected to polymerase chain reaction (PCR) analysis to determine the frequency of colonies positive for proviral DNA.

Fluorescent probe PCR assay (TaqMan)

PCR amplification and subsequent detection of the EYFP and EGFP transgenes was performed by using a quantitative real-time PCR assay (TaqMan). DNA (300 ng) was amplified at least in duplicate with EYFP-specific primers (5'-GGA TTG CAC GCA GGT TCT C-3' and 5'-AGA GCA GCC GAT GTG CTGGT-3') and a fluorescence-tagged probe (5'-FAM-TGG CCA GTC ATA GCC GAA TAG CCT CTC CAT-TAMRA-3'). For EGFP, the specific primers 5'-TAC ACA AAT CCG CCG CAG A-3' and 5'-AGC CTG GTC GAA CCC AGA CAC-3' were used with the probe 5'-FAM-CCA CTA CAC ACC ATG CCG CAG A-3'. These primers and probes were designed using Primer Express software (Perkin-Elmer, Foster City, CA) and were obtained from Synthegen, Houston, TX. Standards consisted of dilutions of DNA extracted from cell lines transduced with a single copy of the EGFP or EYFP vector. Negative controls consisted of DNA extracted from peripheral blood leukocytes (PBLs) from control animals or water. Reactions were run using ABI master mix (Applied Biosystems, Branchburg, NJ) on the ABI Prism 7700 sequence detection system (Applied Biosystems) using 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.
Cytotoxic T-lymphocyte chromium release assays

A T-cell–mediated immune response specific for EGFP/EYFP-expressing cells was assayed in 4 of the 5 animals by cytotoxic T-lymphocyte (CTL) assay. PBMCs were obtained from the peripheral blood of animals by Ficoll density gradient and were cultured with irradiated, autologous MNDEYFPSN-transduced CD34-enriched bone marrow cells at a ratio of 5:1 in RPMI-HEPES 10% HIFBS in the presence of low-dose IL-2 (10 U/mL) for 7 to 15 days. Alternatively, in a few experiments a panel of 20-mer peptides constituting the entire EGFP sequence was used as an antigen source for the in vitro stimulation (kindly provided by Dr Paul Johnson, New England National Primate Center, Southborough, MA). Peptide was added to PBMCs at a concentration of 100 μg/mL total protein (5 μg/mL of each peptide) on day 0 and the cells were cultured as for the stimulation described above. Cytotoxicity of mock-transduced and EYFP-transduced autologous CD34-enriched cells by these in vitro-stimulated PBMCs was assessed in a chromium (Cr-51) release assay. Autologous EYFP-transduced or mock-transduced CD34-enriched bone marrow targets were loaded with Cr-51 overnight and then mixed with the in vitro–stimulated T cells at 1:1, 1:5, and 1:20 ratios in triplicate. Targets were cultured with either medium or NP40 detergent for the determination of minimum and maximum release, respectively. The cultures were incubated for 4 to 5 hours at 37°C, then an aliquot of supernatant was blotted onto a lumaplate for Cr-51 detection. Percent specific lysis was calculated according to the following formula: [(Experimental Release − Minimum Release)/ (Maximum Release − Minimum Release)] × 100. Specific lysis of mock-transduced targets served as an internal negative control for each animal. Other negative controls included parallel CTL assays with PBMCs from a naive animal and an animal tolerant to EGFP/EYFP.

Intracellular cytokine staining assays

The presence of an EGFP/EYFP–specific immune response was also tested by intracellular cytokine staining assay in 2 of the 5 animals. The in vitro–stimulated PBMCs generated by coculture with irradiated, autologous EYFP-transduced CD34+ cells as described above were incubated at 37°C with autologous EYFP-transduced or mock-transduced CD34 cells at a ratio of 1:1 in the presence of anti-CD28 (10 μg/mL; BD Pharmingen) and anti-CD49d (10 μg/mL; BD Pharmingen). After 3 hours, Brefeldin A (10 μg/mL; Sigma) was added to block release of interferon-γ, and the cell suspensions were incubated at 37°C for another 4 hours. After activation, the cells were divided, permeabilized, and stained with anti-interferon-γ–PE (clone 4S.B3; Pharmingen) and CD69–PE (clone SK1; BD Biosciences), and flow cytometric analysis was performed using the FACS Calibur (Becton Dickinson). The forward- and right-angle light scatter–gated events were analyzed to determine the percent of interferon-γ–producing CD8+ cells using CELLQuest v3.3 software. Phorbol 12-myristate 13-acetate (PMA; 10 ng/mL; Sigma)/ionomycin (1 μg/mL; Sigma) and/or staphylococcal enterotoxin B (SEB) stimulation were used as a positive control for each animal assayed. Internal negative controls for each animal consisted of in vitro–stimulated T cells not activated by antigen.

Table 1. Transplantation of oncoretrovirally and lentivirally transduced cells

<table>
<thead>
<tr>
<th>Animal</th>
<th>Vector</th>
<th>Pseudotype</th>
<th>MOI</th>
<th>Growth factors</th>
<th>FACS**</th>
<th>Colonies/1000 cells plated†</th>
<th>Cells infused, *10^6/kg</th>
<th>Days to ANC &gt; 500/μL</th>
<th>Peak marking, %</th>
<th>Lost marking, days</th>
<th>Follow-up, days</th>
</tr>
</thead>
<tbody>
<tr>
<td>M00067</td>
<td>RRLsin.CMVGF (lenti)</td>
<td>VSV-G</td>
<td>10</td>
<td>36SMFG</td>
<td>&lt; 1</td>
<td>25</td>
<td>1</td>
<td>6.1</td>
<td>15</td>
<td>0.1 (11)</td>
<td>54</td>
</tr>
<tr>
<td>RRLsin.cPPT.PGKYFP.Wpre (lenti)</td>
<td>VSV-G</td>
<td>10</td>
<td>36SMFG</td>
<td>3</td>
<td>23</td>
<td>30</td>
<td>0.8 (11)</td>
<td>54</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A00083</td>
<td>RRLsin.cPPT.PGKYFP.Wpre (lenti)</td>
<td>VSV-G</td>
<td>100</td>
<td>36SMFG</td>
<td>52</td>
<td>ND</td>
<td>80</td>
<td>8.2</td>
<td>23</td>
<td>3.6 (14)</td>
<td>35</td>
</tr>
<tr>
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<td>RRLsin.cPPT.PGKYFP.Wpre (lenti)</td>
<td>VSV-G</td>
<td>100</td>
<td>36SMFG</td>
<td>4</td>
<td>25</td>
<td>1</td>
<td>6.1</td>
<td>15</td>
<td>0.1 (11)</td>
<td>54</td>
</tr>
<tr>
<td>A00074</td>
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<td>VSV-G</td>
<td>100</td>
<td>36SMFG</td>
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<td>0.4 (14)</td>
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<tr>
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<td>100</td>
<td>36SMFG</td>
<td>16</td>
<td>ND</td>
<td>ND</td>
<td>9.1</td>
<td>20</td>
<td>0.9 (9)</td>
<td>26</td>
</tr>
<tr>
<td>MNDEYFPPS (onco)</td>
<td>Phoenix–GALV</td>
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<td>36SMFG</td>
<td>17</td>
<td>26</td>
<td>50</td>
<td>19.7</td>
<td>20</td>
<td>1.1 (10)</td>
<td>27</td>
<td>180</td>
</tr>
<tr>
<td>MNDEYFPPS (onco)</td>
<td>Phoenix–GALV</td>
<td>1</td>
<td>36SMFG</td>
<td>6</td>
<td>33</td>
<td>40</td>
<td>17.6</td>
<td>20</td>
<td>0.3 (10)</td>
<td>17</td>
<td>180</td>
</tr>
</tbody>
</table>

MOI indicates multiplicity of infection; S, SCF; M, MGF; F, Flt3-L; G, CSF; ND, not done; ANC, absolute neutrophil count; lenti, lentiviral vector; and onco, oncoretroviral vector. In the column for growth factors, 3 indicates IL-3; and 6, IL-6.

The percentage of EYFP/EYFP-expressing leukocytes in the bulk liquid culture 3 to 5 days after transduction as determined by flow cytometry.

The total number of colonies per 1000 cells plated immediately after transduction.

The percentage of colonies PCR-positive for EYFP/EYFP, indicating the percentage of transduced CFU-Cs in the graft at the time of transplantation.

Results

Transplantation of transduced cells and follow-up

In an attempt to improve the efficiency of HSC transduction, we used a competitive repopulation assay in baboons to study the impact of different culture conditions on the level of in vivo marking achieved using lentiviral vectors. In some of the animals that received lentivirally transduced cells, we observed the complete disappearance of marked cells after an initial engraftment of transgene-expressing cells.19 This finding was in contrast to the results obtained with transplantations we have performed over the past 5 years using stem cells transduced by oncoretroviral vectors where we have always observed some degree of transgene persistence for the duration of our follow-up. To further investigate this finding, we analyzed in more detail 4 of 5 baboons that lost marking to determine whether an immune response to the transgene was responsible for the disappearance of gene-marked cells. Two of the 5 animals received both lentivirally and oncoretrovirally transduced cells, and the other 3 animals received lentivirally transduced cells only. Table 1 summarizes some of the experimental variables as well as the characteristics of the transplantedcell population. Transduction of the bulk CD34-enriched population as assessed by flow cytometry varied from less than 1% to more than 50% and the frequency of proviral-positive CFUs ranged from 1% to 80% as determined by PCR on single colonies. Although the
percentage of CFUs positive for proviral DNA was generally higher than the percentage of bulk culture cells that expressed EGFP/EYFP, the relative marking under the 2 transduction conditions was similar when measured by either method. The animals were followed for approximately 6 months after transplantation (mean follow-up, 180 days).

Transduction of HSCs by VSV-G pseudotype vector does not interfere with engraftment

A possible explanation for the low frequency of transgene-expressing cells detected after transplantation in this study and in studies by An et al.\(^1\)\(^9\)\(^,\)\(^2\)\(^0\) is that the VSV-G envelope protein or another component of the lentiviral vector preparation was toxic to the HSCs and reduced their engraftment ability. To address this possibility, we compared the engraftment kinetics of the 3 baboons that received only VSV-G pseudotype lentivirus-transduced cells (M00067, A00083, and A00074) to the 2 animals who received both oncoretrovirus- and lentivirus-transduced cells (K99307 and M99149) (Table 1), and also to a large group of historic controls that received transplants of EGFP/EYFP oncoretrovirus-transduced cells only. The average days to absolute neutrophil count (ANC) more than 500/\(\mu\)L for the lentivirus-only animals was 19.3 (range, 15 to 23), K99307 and M99149 both engrafted within 20 days, and the mean engraftment in the historic controls was 16.9 days (range, 12 to 28; \(n\) = 11). The similar engraftment kinetics of the cultured repopulating cell populations in these 3 groups of animals suggest that neither the VSV-G envelope nor any other component of the lentivirus preparations was toxic to short-term repopulating cells. In addition, aliquots of the transduced cells from animals A00074, A00083, and K99307 were transplanted into NOD/SCID mice. The engraftment of transduced baboon cells in these mice was similar to engraftment of mock-transduced cells (Figure 1).

**Figure 1.** Engraftment of lentivirus-transduced EGFP/EYFP-expressing baboon CD34-enriched cells in NOD/SCID mice. Displayed are representative flow cytometry data from mice that received transplants in parallel with the autologous baboon transplants. Forward-scatter/side-scatter (FSC/SSC) gating to exclude debris and propidium iodide (PI) gating to exclude dead cells was applied to all plots. (A) Isotype control staining of a mouse that received a transplant of EGFP-expressing lentivirus-transduced A00074 cells (MOI 10) showing GFP-positive events in the lower right quadrant. (B) CD11a-PE staining of the same mouse in panel A showing engraftment of transduced and nontransduced baboon cells. (C) CD11a-PE staining of a mouse that received a transplant of EYFP-expressing lentivirus-transduced A00083 cells (all growth factors). (D) CD11a-PE staining of a mouse that received a transplant of mock-transduced A00074 cells. Given are the percent engraftment (ie, % CD11a\(^+\)) and the percent of CD11a\(^+\) cells expressing EGFP/EYFP.

**Figure 2.** Disappearance of EGFP/EYFP-marked cells after transplantation. Shown for each of the 5 animals that underwent transplantation in this study are the percent of peripheral blood leukocytes positive for transgene as measured by flow cytometry (closed symbols) and by real-time PCR (open symbols). Squares indicate EYFP marking, whereas circles indicate EGFP marking. See Table 1 for vector details.

**Transient expression/gene marking after transplantation**

Although the transduced CD34-enriched cells appeared to engraft normally in these 5 animals based on neutrophil and platelet recovery, the frequency of EGFP/EYFP-expressing cells declined rapidly and disappeared entirely between 26 and 54 days after transplantation. Peak marking of 0.10% to 23.20% was detectable by flow cytometry in these animals (Figure 2).

Generally, the highest marking was seen at the earliest time points after transplantation (9-14 days), and the marking levels decreased dramatically in the subsequent weeks. To determine whether the rapid decline and disappearance of EGFP/EYFP-expressing cells detected by flow cytometry resulted from clearance of these cells from the animal or simply silencing of the provirus, quantitative real-time PCR was performed on DNA extracted from peripheral blood and bone marrow leukocytes. The...
level of marking determined by PCR was slightly higher than that determined by flow cytometry at all time points; however, the dynamics of the marking and expression curves were very similar (Figure 1). All evidence of marking as determined by TaqMan PCR disappeared with the same kinetics as the disappearance of EGFP/EYFP expression detected by flow cytometry, indicating that the gene-modified cells were being cleared, not that the transgene was being silenced.

Detection of EGFP/EYFP-specific cytotoxic T lymphocytes

To determine whether the clearance of gene-marked cells was due to the induction of an immune response against the transgene products, we assayed for the presence of EGFP/EYFP-reactive cytotoxic T cells in the peripheral blood of 4 of 5 animals after transplantation. The fifth animal could not be tested because it was euthanized before the possibility of an immune response was considered, and appropriate samples were not cryopreserved. The CTL assays were performed with in vitro–stimulated fresh or cryopreserved PBMCs drawn 3 to 6 months after transplantation (2-5 months after the loss of marking). We were able to detect the presence of a strong EGFP/EYFP-specific cytotoxic T-cell response by chromium release assay in 3 of the 4 animals and a weak response in the remaining animal (Figure 3). Maximum specific lysis of autologous targets by in vitro–stimulated PBMCs from M00067 (A), K99307 (B), A00083 (C), M99149 (D), EGFP/EYFP-tolerant transplanted control animal M99267 (E), and naive control animal T99151 (F). Assays were done in triplicate at 3 effector-to-target (E/T) ratios as indicated.

Figure 3. Detection of EGFP/EYFP-specific cytotoxic T-lymphocyte responses. Percent specific lysis of mock-transduced (○) or EYFP-transduced (□) autologous targets by in vitro–stimulated PBMCs from M00067 (A), K99307 (B), A00083 (C), M99149 (D), EGFP/EYFP-tolerant transplanted control animal M99267 (E), and naive control animal T99151 (F). Assays were done in triplicate at 3 effector-to-target (E/T) ratios as indicated.

active phase of rejection. To ensure that we were detecting a memory response and not priming a response against EGFP/EYFP by our in vitro stimulation conditions, we performed identical assays with PBMCs from a naive animal (T99151) and a transplant animal with stable EGFP/EYFP marking of more than 8% more than 1 year after transplantation (M99267). The cultures from these control animals did not show any specific lysis of EGFP/EYFP-expressing autologous targets. As additional evidence of the presence of an immune response against the transgene product, we were able to detect EGFP/EYFP-specific CD8+ T lymphocytes in in vitro–stimulated peripheral blood of 2 of the animals by staining for intracellular interferon–γ after brief stimulation with autologous EYFP-transduced cells (Figure 4). In the majority of experiments, the autologous CD34-enriched cells used as antigen presenting cells (APCs) for the in vitro stimulation and as targets for the CTL assays were transduced with the oncoretroviral vector MNDEYFPSN. From animals that received transplants of only lentivirally transduced cells, we assayed for the presence of EGFP/EYFP-reactive T cells in the peripheral blood of 4 of 5 animals after transplantation. The cultures from these animals did not show any specific lysis of EGFP/EYFP-transduced cells even after in vitro stimulation and as targets for the CTL assays were transduced with the oncoretroviral vector MNDEYFPSN. The fact that we were able to detect cytotoxic activity in PBMCs derived from the EGFP- or EYFP-transduced autologous targets confirms that the immune responses we were assaying were specific for the EGFP/EYFP transgene product and not for cryptic antigens expressed in the lentiviral vector or for any other protein components of the vector preparation which may have been transiently transferred to the repopulating cells during the transduction culture. In 2 of the animals, we were also able to detect specific killing when an EGFP peptide panel was the antigen source, corroborating that the immune responses are against epitopes derived from the EGFP or EYFP protein (data not shown). Given that EGFP and EYFP differ by only 4 of 240 amino acids, it is likely that any responses mounted against cells expressing one protein would be capable of killing cells expressing the other protein.

Phenotype of lentivirus- and oncoretroviral-transduced cells

Because we have only observed the induction of transgene-specific immune responses after myeloablative TBI in animals that received transplants of lentivirally transduced cells, we compared our oncoretroviral and lentiviral transduction cultures to determine if the cell populations differed in the expression of differentiation markers or in the EGFP/EYFP transduction of specific subsets of
Discussion

In the current study we demonstrate for the first time the induction of transgene-specific CTL responses to gene-modified cells after a myeloablative conditioning regimen in baboons. These immune responses were detected after transplantation of CD34-enriched cells transduced with lentiviral vectors alone or in combination with oncoretroviral vectors and resulted in the complete disappearance of gene-modified cells. Our data suggest that the use of lentiviral vectors may contribute to the development of antitransgene immune responses in these animals.

A potential explanation for the low marking in our animals could be a toxic effect of the VSV-G envelope protein or some other component of the virus preparations on the engrafting cells. VSV-G protein has been shown to be toxic to numerous cell types. While this was a concern in our study, the timely recovery of neutrophil counts after transplantation in the 5 animals reported here and especially in the 3 animals receiving only VSV-G pseudotype lentivirally transduced cells suggests that the lentiviral vectors did not have a toxic or damaging effect on repopulating cells. The lack of toxicity on repopulating cells was also supported by the finding that there was no negative effect of VSV-G-transduced cells on engraftment in the NOD/SCID model. Thus, the use of the VSV-G pseudotype was not responsible for the disappearance of marking. As additional evidence of lack of toxicity, mock-transduced VSV-G pseudotype lentivirus-transduced and GALV pseudotype oncoretrovirus-transduced populations had similar expansion and CFU clonogenicity (Table 1), indicating comparable maintenance of progenitors during transduction with the different vectors.

Whereas our long-term marking levels after oncoretroviral transductions vary from very low (<1% of PBLs) to high (>20%), we have never observed complete disappearance of gene-modified cells in these studies. We have performed transplantations on more than 14 baboons with oncoretrovirally transduced cells encoding EGFP/EYFP, and all 14 animals showed long-term persistence of EGFP/EYFP-expressing cells after transplantation, as measured by both flow cytometry and quantitative PCR (marking range: 0.4% to 18.8% by FACS; follow-up range: 1.5 to 42 months). In contrast, in the current study, 5 of 5 animals that received lentivirally transduced cells completely lost marking. Based on our experience with immune responses after nonmyeloablative transplants, we hypothesized that immune responses against EGFP/EYFP could be responsible for this disappearance of gene-modified cells. In 4 of these 5 animals we were able to document T-cell–mediated immune responses against EGFP/EYFP by both cytotoxicity assay of in vitro–cultured T cells and intracellular cytokine staining of peripheral blood T cells. Identical assays from a tolerant animal (M99267) and a naive animal (T99151) proved that our assay conditions were not capable of priming a response in vitro and that we were detecting a memory response.

The disappearance of marked cells due to an immune response was previously shown to occur after nonmyeloablative conditioning (240 cGy TBI). Our current results show that immune responses to transduced cells can also occur after myeloablative conditioning (1020 cGy TBI). The T cells causing the disappearance of gene-marked cells after myeloablative conditioning may originate from (1) T cells infused together with the CD34 cell graft; (2) grafted hematopoietic cells differentiating into T cells in the thymus; or (3) T cells that survived the conditioning. The origin from T cells infused with the graft is unlikely since we detected no T cells in the grafts by flow cytometry (suggesting that <5 × 10^3 T cells/kg were infused, assuming 0.1% sensitivity of flow cytometry). The origin from grafted hematopoietic cells is also unlikely since in baboons T cells start to be generated de novo after 2 months after transplantation, whereas the disappearance of the marked cells occurred between day 26 and day 54. Thus, the T cells causing the disappearance likely originate from T cells that survived the 1020 cGy TBI. Consistent with that, there appears to be a marked proliferation of the surviving T cells in the first month after transplantation so that by day 28, CD4 T-cell counts reach approximately 30% of pretransplant levels, and CD8 T-cell counts near 100% pretransplant levels (Storek et al); and J. S. and H.-P. K., unpublished data, August 2003). Given that T cells that survive 1020 cGy TBI likely take part in the immune response...
levels of overall marking may induce peripheral tolerance in inducible tolerance to transgenes, and if this level is not achieved, some investigators have suggested that there is a threshold level of marking necessary to and not oncoretrovirally transduced cells? Some investigators have

be avoided by a more T-cell against the transgene-expressing cells, the immune response might potent immune responses to the EGF/VEGF transgenes despite the use of a myeloablative conditioning regimen. The use of lentiviral vectors to transduce cells may play a role in the development of immune responses in this setting. These results have important implications for the development of gene therapy protocols.

Another possibility for the induction of immune responses after infusion of lentivirally versus oncoretrovirally transduced cells could be that the lentiviral vectors induce differentiation of the CD34-enriched population such that there are more immune response promoting cells or fewer tolerizing cells at the end of the culture period. Our analysis of differentiation marker expression on transduction cultures in 4 independent in vitro experiments suggests that the oncoretroviral and lentivirally transduced cell populations are not significantly different in their expression of CD3(T cell), CD13 and CD14 (granulocyte/monocyte), CD20 (B cell), CD34 (progenitor), or CD83 (mature dendritic cell)—and not different from mock-transduced cultures—and therefore our vectors are not driving differentiation differently. However, when we analyzed EYFP expression mediated by lentiviral or oncoretroviral vectors in these subsets, we found significant differences in transduction rates. The oncoretroviral vectors transduced the whole leukocyte (PBL), CD13, CD34, and CD83 populations at a significantly higher rate than the lentiviral vectors. The difference was particularly remarkable for CD34+ cells, which were transduced about 5 times more efficiently by oncoretroviral vectors than by lentiviral vectors. Although not statistically significant (P = .15), the phenotyping data suggests that the lentiviral transduction of CD34+ cells is lower than that of the whole leukocyte population. Other investigators have suggested that sustained expression of foreign gene products in HSCs may be critical for the induction of tolerance.30-32 Perhaps the transduction rate into CD34+ cells by our lentiviral vectors was not sufficient to induce tolerance. In that case, the use of recently described modified HIV-based lentiviral vectors may circumvent this problem.33

In conclusion, we have shown that baboons that received transplants of gene-marked bone marrow cells have developed potent immune responses to the EGF/VEGF transgenes despite avascular bone marrow: implications for gene therapy. Nat Med. 1996;2:1305-1312.

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References


Induction of cytotoxic T-lymphocyte responses to enhanced green and yellow fluorescent proteins after myeloablative conditioning

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