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Repopulation of B-lymphocytes with restricted gene expression using haematopoietic stem cells engineered with lentiviral vectors

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Abstract

B-lymphocytes play a key role in the pathogenesis of many immune-mediated diseases, such as autoimmune and atopic diseases. Therefore, targeting B-lymphocytes provides a rationale for refining strategies to treat such diseases for long-term clinical benefits and minimal side effects. In this study we describe a protocol for repopulating irradiated mice with B-lymphocytes engineered for restricted expression of transgenes using haematopoietic stem cells. A self-inactivating lentiviral vector, which encodes enhanced green fluorescence protein (EGFP) from the spleen focus-forming virus (SFFV) promoter, was used to generate new vectors that permit restricted EGFP expression in B-lymphocytes. To achieve this, the SFFV promoter was replaced with the B-lymphocyte-restricted CD19 promoter. Further, an immunoglobulin heavy chain enhancer (E μ) flanked by the associated matrix attachment regions (MARs) was inserted upstream of the CD19 promoter. Incorporation of the E μ -MAR elements upstream of the CD19 promoter resulted in enhanced, stable and selective transgene expression in human and murine B-cell lines. In addition, this modification permitted enhanced selective EGFP expression in B-lymphocytes in vivo in irradiated mice repopulated with transduced bone marrow haematopoietic stem cells (BMHSCs). The study provides evidence for the feasibility of targeting B-lymphocytes for therapeutic restoration of normal B-lymphocyte functions in patients with B-cell-related diseases.

Keywords

B-lymphocyte; lentiviruses; stem cells

Introduction

B-lymphocytes play a key role in chronic inflammation and in the pathogenesis of many diseases such as autoimmunity and allergy. In autoimmune diseases, B-lymphocytes produce pathogenic autoantibodies and disease-promoting cytokines and can activate autoreactive T cells. The use of B-cell depleting anti-CD20 antibodies has proved to be therapeutically effective in a variety of autoimmune diseases such as rheumatoid arthritis,

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systemic lupus erythematosus and multiple sclerosis.¹ The successful application of anti-CD20 in the clinic has strengthened the argument that B cells play a key role in the pathogenesis of many autoimmune and inflammatory diseases and provided a rationale for more refined means of manipulating B-lymphocyte responses in these conditions.²⁻⁵ This approach would be helped by the identification of key molecules that regulate B-lymphocyte responses through the generation of knockout and transgenic mice in which diseases are either significantly delayed, incidence reduced or completely abrogated.⁶

B-lymphocytes contribute to the pathogenesis of autoimmune diseases through antibody-dependent and -independent mechanisms. Autoantibodies form immune complexes that activate the complement system and induce pro-inflammatory cytokines. In addition, autoantibodies couple the adaptive humoral response to innate effector pathways, such as phagocytosis, antibody-dependent cellular cytotoxicity, and the recruitment and activation of inflammatory cells by binding to Fc γ receptors.⁷ B-lymphocytes can also produce cytokines such as interferon- α , IL-6 and IL-10, all of which further promote inflammation and autoantibody production.¹ In addition, B-lymphocytes can present autoantigens to and activate autoreactive T-lymphocytes.⁸ Although depleting B-lymphocytes with anti-CD20 antibody for the treatment of patients with B-cell lymphomas and some autoimmune diseases has been highly successful there are also potential limitations.^{5,9} For example, there is new evidence for increased risk of fatal viral infections.¹⁰ In addition, B-lymphocyte depletion is not a cure and clinical benefits are mostly symptomatic. Further, not all patients benefit clinically and in some the benefits are temporary.⁵ Therefore, targeting specific molecules, or signalling pathways that regulate the responsiveness of autoreactive B-lymphocytes could in the long term provide better options for treatment.

Haematopoietic stem cell-based gene therapy has been shown to be a promising strategy for treating inherited haematopoietic disorders. In autoimmune diseases, haematopoietic stem cells could also be ideal tools for manipulating lymphocytes because they can reconstitute the entire immune system in the recipient. Gene therapy targeting specific pathways in haematopoietic stem cells has been successfully applied for the treatment of patients with cancer and patients with inherited immunodeficiency such as X-linked, recessive SCID (caused by common cytokine-receptor γ -chain deficiency) and patients with adenosine-deaminase deficiency.¹¹⁻¹⁴ Gene therapy delivered by lentivirus targeting haematopoietic stem cells offers several advantages over other methods of treatment.¹⁵ These include long-term, *in vivo* production of therapeutic molecules through permanent integration into the genome of quiescent cells with life-long capacities for extensive proliferation and differentiation. In addition, lentivirus-based gene therapy provides the possibility that large genomic elements, such as promoters and enhancers, could be inserted into the transducing vector.¹⁶ Further, they provide tools for lineage-specific expression. For example, lentiviral vectors have been successfully used for lineage-specific transgene expression in erythrocytes, T-lymphocytes and in dendritic cells.^{17,18}

Self-inactivating lentivirus vectors have been shown to be efficient in transgene expression from an internal promoter and/or other regulatory elements. However, transgene expression in different cell types, or in all progenies of the transduced haematopoietic stem cells is often unnecessary and could lead to adverse effects. Therefore, targeted and selective expression of transgenes, or therapeutic elements in one cell type, or one lineage of haematopoietic stem cells will be crucial to achieve maximal therapeutic benefits and reduce potential adverse effects. Selective transgene expression in erythrocytes was achieved by using the regulatory elements that promote erythrocyte-specific genes and has proven to be successful in treating β -thalassaemia.^{16,19,20}

B-lymphocyte-specific expression of transgenes was previously achieved by incorporating the CD19 regulatory elements in a retroviral system.²¹ Furthermore, it was shown that incorporating regulatory elements from the immunoglobulin locus, such as the heavy chain intron enhancer ($E\mu$), leads to enhanced transgene expression in B-lymphocytes.²²⁻²⁴ However, the degree of enhancement in these studies was shown to be variable. Consistent, position-independent expression in B-lymphocytes was achieved by flanking the $E\mu$ sequence with matrix attachment regions (MARs).²⁴ MARs can affect expression by altering the organization and structure of chromatin domains, maintenance of a domain of chromatin accessibility and enhancing histone acetylation.^{25,26} The $E\mu$ -MARs elements could possibly influence transgene expression by two mechanisms. First, $E\mu$ -MARs possess binding sites for transcription factors, such as Bright (B-cell regulator of immunoglobulin heavy chain transcription), which enhances expression in activated, or terminally differentiated B-lymphocytes.²⁷ Second, $E\mu$ -MARs have repressor elements, such as SATB1, that prevent expression in non-B-lymphocytes such as T-lymphocytes.²⁸

In this study, we attempt to develop a protocol for targeting transgene expression to B-lymphocytes. We used $E\mu$ -MAR and CD19 promoter elements to produce consistent and selective expression of enhanced green fluorescence protein (EGFP) from lentiviral vectors in B-lymphocytes differentiated from bone marrow haematopoietic stem cells (BMHSCs). Further, we demonstrate long-term transgene expression in mice transplanted with transduced haematopoietic stem cells.

Results

Insertion of $E\mu$ -MAR upstream of the PGK promoter promotes transgene expression in various cell types

A schematic diagram for the lentiviral vectors used in our experiments is given in Figure 1. To evaluate the efficiency and specificity of EGFP transgene expression using the different lentiviral vectors we first tested the SFFV-EGFP and $E\mu$ -MAR-PGK-EGFP vectors in a B-lymphocyte cell line (Ramos) and non-B-lymphocyte cell lines (Jurkat T-cell lines and HEK-293T fibroblastic cell line). The cells were exposed to the lentiviruses in the presence of Polybrene ($8 \mu\text{g ml}^{-1}$) for 4 days. The lentiviral vector $E\mu$ -MAR-PGK-EGFP, which contains the $E\mu$ -MAR elements upstream of the phosphoglycerate kinase (PGK) promoter, has been shown to enhance transgene expression in B-lymphocytes.²⁴ Flow cytometric analyses of EGFP expression showed that both the SFFV-EGFP and $E\mu$ -MAR-PGK-EGFP vectors induced EGFP expression in B- and non-B-lymphocyte cell lines (Figure 2). As shown in Figure 2, SFFV-EGFP induced EGFP expression in 19% of the Ramos B cell line, 58% Jurkat T cells and 52% HEK-293T cells. The $E\mu$ -MAR-PGK-EGFP increased the percentage of Ramos B cells expressing EGFP to 41% and 95% of Jurkat T cells and 83% of HEK-293T cells. Thus, the $E\mu$ -MAR upstream of the PGK promoter did not restrict EGFP expression to the B-lymphocyte cell line.

Replacement of the SFFV promoter with CD19 restricts transgene expression to B-lymphocytes

A new set of lentiviral vectors was generated by replacing the spleen focus-forming virus (SFFV) promoter either with the B-lymphocyte-specific promoter CD19 alone, or together with the $E\mu$ -MAR elements. The effect of incorporating the CD19 promoter with and without the $E\mu$ -MAR on EGFP transgene expression in B-lymphocytes was investigated by flow cytometry. The data showed that both lentiviral vectors, CD19-EGFP and $E\mu$ -MAR-CD19-EGFP induced EGFP expression in both human and murine B-cell lines (Ramos and LK-35) but not in T-cell lines (Jurkat and BW5147). In three separate experiments for each cell line the CD19-EGFP induced EGFP expression in 16.7 ± 3.4 per cent (mean $\pm$ s.e.m.)

Ramos cells and $11.0 \pm 1.5\%$ of LK-35 cells with mean fluorescence intensity (MFI) of 40 (Figure 3). In the Jurkat and BW5147 T-cell lines, EGFP expression with the CD19-EGFP vector were 2.3 ± 0.3 and $1.3 \pm 0.3\%$, respectively, with a background MFI of <5 . Transduction with the E μ -MAR-CD19-EGFP vector in three separate experiments resulted in EGFP expression in $18.3 \pm 1.8\%$ of Ramos cells and $21.7 \pm 2.2\%$ of LK-35 cells with an average MFI of about 200. Expression of EGFP in the Jurkat and BW5147 cell lines transduced with this vector were 3.3 ± 0.7 and $4.3 \pm 0.3\%$, respectively with a background MFI of <5 . As expected, the SFFV-EGFP lentiviral vector induced EGFP expression in both B- and T cells. Transduction with this vector resulted in EGFP expression in $21.7 \pm 2.9\%$ Ramos cells, $20.0 \pm 1.5\%$ LK-35 cells, $58.3 \pm 7.3\%$ Jurkat T cells and $56.7 \pm 4.9\%$ BW5147 murine T-cell line (Figure 3).

The data, thus, confirms that the presence of the CD19 promoter leads to selective transgene expression in B-lymphoid lineage cells. Additionally, the presence of E μ -MAR moderately increased the percentage of cells transduced and significantly enhanced the level of EGFP expression by 3–5 folds in B cells without reducing specificity of transgene expression.

Reconstitution of irradiated mice with lymphocytes using transduced haematopoietic stem cells

To investigate the feasibility of restricted transgene expression *in vivo*, we first established and assessed protocols for the isolation, culture and transduction of BMHSCs *in vitro*. BMHSCs were first isolated as Lin[−] cells from the washout of murine bone marrow cells using Lin[−] isolation kit and cultured in X-VIVO-15 medium conditioned with detoxified bovine serum albumin (BSA), SCF, FLT-3 ligand and IL-7. Two weeks after initiation of the culture, about 14% of the generated cells were assessed to be phenotypically of the B-lineage cells (data not shown). This data verified the isolation procedure but the procedure proved to be inefficient in generating B-lymphocytes expressing EGFP. The protocol was, therefore, modified and Lin[−] BMHSCs cells were cultured on Retronectin-coated tissue culture plates in I-MDM supplemented with fetal bovine serum (FBS), murine IL-3, SCF and human IL-6. This modification resulted in significantly higher percentage cell transduction with $>30\%$ of the resulting cells expressing EGFP. This modified protocol was thereafter used for transduction and culturing BMHSCs in preparation for transplantation *in vivo*. Generally, two millions of transduced BMHSCs were transplanted into lethally irradiated 8-week-old Balb/c mice and repopulation of lymphocyte assessed. For the experiments shown in Figure 4 two groups of four mice each were analysed. Both groups were lethally irradiated with one group not receiving BMHSCs to serve as control for the irradiation procedure while the second group was transplanted with 2×10^6 nontransduced BMHSCs per mouse. All irradiated mice that did not receive BMHSCs died within 10 days of the irradiation. Just before their death, peripheral blood from these control mice were collected and tested for the presence of lymphocytes. Figure 4 shows that no, or very few, lymphocytes were detectable in the blood of the irradiated control mice while mice in the group reconstituted with the BMHSCs were reconstituted with B- and T-lymphocytes. Before irradiation, $49.8 \pm 0.9\%$ of blood cells within the lymphocyte gate in experimental mice were CD4⁺ T-lymphocytes while $22.3 \pm 0.5\%$ were CD19⁺ B-lymphocytes. Two months after reconstitution with transduced BMHSCs, distribution of blood cells within the lymphocyte gate of the second group of mice were $51.0 \pm 2.2\%$ CD4⁺ T-lymphocytes and $22.8 \pm 1.4\%$ CD19⁺ B-lymphocytes.

EGFP transgene expression in primary B-lymphocytes

To test levels and restriction of EGFP expression *in vivo*, four groups of four 8-week old Balb/c mice each were irradiated and either left without reconstitution (one group), or reconstituted with 2×10^6 BMHSCs transduced with SFFV-EGFP, CD19-EGFP or E μ -

MAR-CD19-EGFP vectors. All mice from the lethally irradiated group that were not reconstituted with BMHSCs died within 10 days. Mice from the other three groups survived and were studied for lymphocyte repopulation and EGFP expression. Peripheral blood samples were collected at 2 and 3 months post transplantation and nucleated blood cells analysed for EGFP expression in CD19⁺ B-lymphocytes and in CD19⁻ cells (non-B cells; include all blood cells within the lymphocyte gate excluding CD19⁺ B-lymphocytes) by FACS. In addition, at 3 months the mice were culled and spleens collected and analysed. Percentages of B- and non-B cells expressing EGFP and levels of expression in the blood of recipient mice at 2 months and spleens at 3 months post transplantation are shown in Figure 5. Percentages of B- and non-B cells expressing EGFP remained constant at 2 and 3 months (not shown) and were similar to those in the spleen at 3 months. The SFFV-EGFP vector induced EGFP expression in $8.5 \pm 0.3\%$ blood B-lymphocytes and in $10.5 \pm 0.6\%$ non-B cells at 2 months post transplantation (Figure 5a). In splenocytes, 8.3 ± 0.8 B-lymphocytes and 10.5 ± 0.9 non-B cells within the lymphocyte FACS gate expressed EGFP. In contrast, in mice transplanted with BMHSCs transduced with CD19-EGFP, or E μ -MAR-CD19-EGFP, EGFP was clearly detectable in B-lymphocytes but not in non-B-lymphocytes. Thus, in mice transplanted with CD19-EGFP-transduced BMHSCs 5.5 ± 0.3 and $4.5 \pm 0.3\%$ blood and spleen (at 3 months) B-lymphocytes, respectively, expressed EGFP. In contrast, only $0.3 \pm 0.06\%$ of blood non-B cells and 0.4 ± 0.06 spleen non-B cells had very low levels of EGFP. In mice transplanted with BMHSCs transduced with E μ -MAR-CD19-EGFP 3.8 ± 0.3 blood and 2.5 ± 0.3 spleen B cells expressed EGFP compared with 0.3 ± 0.04 blood and 0.3 ± 0.05 spleen non-B cells. B-lymphocytes in the group of mice transplanted with E μ -MAR-CD19-EGFP transduced BMHSCs had on average sevenfold increase in the expression level of EGFP compared with mice transplanted with CD19-EGFP/BMHSC; 90.8 ± 1.2 compared with 12.5 ± 1.4 MFI. When the results were analysed as per cent B- and non-B-lymphocytes within the gated EGFP⁺ cells, 24.3 ± 5.8 , 93.5 ± 0.9 and $93.0 \pm 1.2\%$ were B-lymphocytes and 75.8 ± 5.8 , 6.5 ± 0.9 and $7.0 \pm 1.2\%$ were non-B cells in mice transplanted with BMHSCs transduced with SFFV-EGFP, CD19-EGFP and E μ -MAR-CD19-EGFP vectors, respectively. The results for splenocytes were 42.3 ± 8.0 , 91.5 ± 0.9 and $92.0 \pm 1.2\%$ B-lymphocytes and 57.8 ± 8.0 , 8.5 ± 0.9 and $8.0 \pm 1.2\%$ non-B cells in mice transplanted with BMHSCs transduced with SFFV-EGFP, CD19-EGFP and E μ -MAR-CD19-EGFP vectors, respectively.

Variations between individual mice in percentage lymphocytes expressing EGFP were relatively low. Thus, coefficient of variation (COV) for EGFP expression in blood B-lymphocytes were 6.7, 10.4 and 13.3 in mice recipients of BMHSCs transduced with SFFV-EGFP, CD19-EGFP and E μ -MAR-CD19-EGFP, respectively.

Discussion

In the last few years, depletion of B-lymphocytes has been successfully used in the clinic to treat patients with a variety of B-cell-related diseases such as B-cell lymphomas and autoimmune diseases.¹ However, there is evidence that the approach could have serious side effects such as increased susceptibility to viral infections.¹⁰ The development of protocols whereby selected molecules and/or pathways in B-lymphocytes, or relevant subpopulations could be targeted could provide major improvements in treatment. Gene therapy has been used to introduce 'therapeutic' genes for the treatment of a number of cancers. However, the introduction of genes to rectify selected functions in B-lymphocytes, or to target subpopulations thereof, such as autoreactive B-lymphocytes poses considerable challenges. Lentiviruses have been shown to be effective and efficient in transducing and permitting transgene expression in different cell types.¹⁵ Self-inactivating lentiviruses in which the viral promoter is disabled by deleting the enhancers and TATA box in the U3 promoter region of the 3' LTR and transgene is regulated through internal promoter provide new tools

for long-term expression of the therapeutic molecules.²⁹ Furthermore, incorporating a tissue specific promoter permits selective expression of the therapeutic agents in one cell type, or one lineage of haematopoietic cells. This is crucial to achieve maximal therapeutic benefits and reduce adverse side effects. Lineage-specific expression will also be required for some disorders affecting specific cells, or single lineages. Noteworthy, erythroid-specific lentiviral vectors expressing the β -globin gene were successfully used to correct the β -thalassemic phenotype.¹⁹

Expression of EGFP transgene selectively in B-lymphocytes was previously achieved by incorporating the CD19 promoter into a self-inactivating retroviral vector.²¹ However, these investigators observed that the level of transgene expression was relatively low and inconsistent. Our experiments in which we inserted the E μ -MAR elements into the lentiviral vector upstream of the CD19 promoter showed that the modification resulted in enhanced, consistent and restricted EGFP expression in B-lineage cells. This is in agreement with previous investigations in which the E μ -MAR elements inserted into lentiviral vectors resulted in enhanced and consistent expression of EGFP in B-lymphocytes.²⁴ However, the expression was not restricted to B-lymphocytes.

We first evaluated expression profiles of different lentiviral vectors. As shown in Figure 2, both SFFV-EGFP and E μ -MAR-PGK-EGFP vectors induced transgene expression in all cell lines tested. The presence of E μ -MAR upstream of the PGK promoter did not restrict EGFP expression to B-lymphocytes. In the second set of experiments, we evaluated the effect of inserting the CD19 promoter alone, or with the E μ -MAR on transgene expression. As shown in Figure 3, transduction with both vectors CD19-EGFP and E μ -MAR-CD19-EGFP resulted in selective transgene expression in human and murine B-cell lines (Ramos and LK-35, respectively) but not in T-cell lines (Jurkat and BW5147). Insertion of the E μ -MAR elements upstream of the CD19 promoter enhanced transgene expression in B-lymphocytes.

To assess level, consistency and restriction of expression *in vivo* we studied EGFP expression in lethally irradiated mice reconstituted with transduced BMHSCs. As shown in Figure 5, the CD19-EGFP and E μ -MAR-CD19-EGFP vectors selectively induced EGFP expression in reconstituted B-lymphocytes, but not in T-lymphocytes. Further, despite sustained repopulation of B-lymphocytes with newly generated cells, our results showed that EGFP expression persisted at almost the same levels until termination of the experiments 3 months post transplantation of the engineered BMHSCs.

The results also confirmed that the level of EGFP expression was enhanced in B-lymphocytes in mice recipient of BMHSCs transduced with the E μ -MAR-CD19-EGFP vector compared with cells transduced with CD19-EGFP. Enhancement of transgene expression in the presence of the E μ enhancer could be due to binding of the enhancer to transcriptional factors present in cells of the B-lymphoid lineage, such as Bcl-6.²⁷ These results are consistent with previous studies in which the E μ enhancer was shown to enhance transgene expression in B-lymphocytes *in vivo* and *in vitro*.²²⁻²⁴

Recombinant lentiviral vectors integrate into different sites of the genome in target cells. Thus, expression from integrated lentivirus vectors may be influenced by transcriptional enhancers, silencers or other regulatory elements in the local chromatin environment, leading to inconsistent, or variable expression from each integration site. There is evidence for a structural role for MAR in dividing eukaryotic chromosomes into topologically looped domains that protect transgenes against transcription silencing.³⁰ It has also been shown that MAR elements can influence expression by affecting the organization and structure of chromatin domains, chromatin accessibility and enhancing histone acetylation.^{25,26} The

MAR elements integrated into our lentiviral constructs could also have influenced EGFP expression through possessing binding sites for transcription factors, such as Bright. Bright enhances gene expression in activated and terminally differentiated B-lymphocytes.²⁷ Alternatively, MAR may have enhanced EGFP expression by suppressing repressor genes such as SATB1, which has been shown to operate in lymphocytes.^{27,28}

In studies of transgenic mice, position-independent expression in B-lymphoid lineage cells from expression cassettes with the E μ enhancer was only observed if the MARs, which flank the E μ enhancer, were included in the vector. Lutzko *et al.*²⁴ evaluated uniformity of transgene expression from integrated lentiviruses by comparing the COV for the level of EGFP expression in circulating murine B- and non-B-lymphoid cells transduced with lentiviruses with and without the E μ and E μ -MAR elements. Lower COVs were observed in B-lymphocytes than in non-B-lymphocytes in mice recipients of the E μ -MAR-PGK vector. When the E μ -PGK vector was used without the MAR element high COVs were also observed in B-lymphocytes. These investigators also demonstrated that the E μ -MAR elements are necessary for uniform, position-independent B-lymphoid transgene expression from lentiviral vectors.²⁴ In addition, studies in transgenic mice by Jenuwein *et al.*²⁵ suggested that the E μ -MAR elements maintained the chromatin in an open configuration and thereby accessible to transcriptional machinery.

In summary, this study shows that presence of the E μ -MAR element upstream of PGK promoter results in enhanced expression of EGFP transgene in B-lymphocytes. However, EGFP expression was not restricted to B-lymphocytes. Incorporation of the E μ -MAR elements upstream of the CD19 promoter in lentivirus vectors resulted in enhanced, stable and selective transgene expression in human and murine B cells. In addition, selective and enhanced transgene expression in B-lymphocytes was observed from integrated lentiviruses expressing from the CD19 promoter with the E μ -MAR element in primary murine cells differentiated from transduced progenitor BMHSCs *in vivo*. The development of these lentiviral tools could benefit the development of therapeutic approaches to modulate humoral autoimmunity by selectively targeting B-lymphocytes.

Materials and methods

Cell lines and conjugated antibodies

The human Burkitt's lymphoma B-cell line Ramos, murine B-cell line LK-35, human Jurkat T cells, murine T-cell line BW5147 and epithelial cell line HEK-293T cells were from the ATCC (Manassas, VA, USA). All cell lines, except HEK-293T cells, were cultured in RPMI 1640 containing L-glutamine supplemented with 10% fetal calf serum (FCS) and a mixture of penicillin, streptomycin and amphotericin B (HybriMax mix, Sigma-Aldrich, Dorset, UK). HEK-293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and the antibiotic mix. Phycoerythrin (PE)-conjugated rat anti-mouse CD19 (anti-CD19-PE), anti-mouse CD4 (anti-CD4P-PE) and control conjugated antibody isotypes were obtained from BD Biosciences (Oxford, UK).

Lentiviral vector construction and lentivirus production

The lentiviral vectors used in this study are based on the self-inactivating vector pHR SIN-CSGW-dNotI (SFFV-EGFP) kindly provided by Dr Y Ikeda (Mayo Clinic, Rochester, MN, USA). The CCL-EMP-EGFP (E μ -MAR-PGK-EGFP) plasmid was kindly provided by Dr C Lutzko (Children's Hospital, SABAN Research Institute, Los Angeles, CA, USA). The viral promoters in the lentiviral vectors were disabled by deleting the enhancers and TATA box in the U3 promoter region of the 3' LTR. Two basic vector designs were used with the EGFP reporter transgene expressed from either murine phosphoglycerate kinase promoter (E μ -

MAR-PGK-EGFP), or human spleen focus-forming virus promoter (SFFV) (SFFV-EGFP, Figure 1). In some lentiviral vectors, a fragment containing the core CD19 promoter (from the retroviral vector SIN-CD19-W; kindly provided by Professor T Brocker, Ludwig-Maximilians-Universität, München, Germany), was inserted into the lentiviral vectors replacing the SFFV promoter. In other vectors, the fragment from the Ig heavy chain locus which includes E μ and its flanking MARs (E μ -MAR), kindly provided by Marc Shulman (University of Toronto), was inserted upstream of the CD19 promoter. The lentiviruses thus generated were produced by triple transfection of HEK-293T cells as described.³¹ Briefly, 18 μ g of the transducing plasmid, 18 μ g of the pCMVR8.2 packaging plasmid and 4.5 μ g of pMDG plasmid encoding VSV-G envelope were transfected into ~40% confluent HEK-293T cells (in 10-cm-diameter Petri dishes). Supernatants were collected during the 96 h post transfection and concentrated by ultracentrifugation.

Cell line transduction with lentiviruses

Two hundred thousand cells in 1 ml medium were incubated with the lentiviruses in the presence of 8 μ g ml⁻¹ of Polybrene (Sigma-Aldrich). Four days after transducing the cells, percentages of EGFP-expressing cells were determined by flow cytometry.

Murine bone marrow haematopoietic stem cell harvesting, transduction and transplantation

Balb/c mice were purchased from Harlan-UK (Loughborough, UK) and maintained at the Biological Services Unit at Charterhouse Square, Queen Mary School of Medicine and Dentistry. Donor bone marrow cells were harvested from the femur of 8-week-old male Balb/c mice by flushing with phosphate-buffered saline (PBS) containing 2% FCS 2 days after injecting the mice with 150 mg kg⁻¹ body weight 5-fluorouracil (Sigma-Aldrich). The protocol for isolating and culturing BMHSCs was first evaluated by assessing their ability to differentiate and be transduced *in vitro*. To achieve this, Lin⁻ cells were isolated from the bone marrow washouts using Lin⁻ isolation kit (StemCell Technologies SARL, London, UK) and cultured in X-VIVO-15 medium conditioned with detoxified BSA, SCF, FLT-3 ligand and IL-7. The cells were cultured for 2 weeks and differentiation analysed by flow cytometry. The experiments showed that 14% of the differentiated cells after 2 weeks were B-lineage cells (CD19⁺). This suggested that the isolation procedure and differentiation were successful. However, transduction of the Lin⁻ BMHSCs cultured according to this protocol was inefficient as only a small proportion of the generated B cells expressed EGFP. The protocol was, therefore, modified and BMHSCs were cultured at on Retronectin-coated tissue culture plates in I-MDM supplemented with 20% FBS, 10 ng ml⁻¹ murine IL-3, 2.5 ng ml⁻¹ murine SCF (Biosource Europe, Nivelles, Belgium), 50 ng ml⁻¹ human IL-6 (hIL-6), 2 mM L-glutamine, HybriMax antibiotic mix and 10⁻⁵ M 2-mercaptoethanol. The BMHSCs were cultured for 2 weeks and differentiation and EGFP expression determined. Under these conditions >30% of the generated lymphocytes expressed EGFP. For preparing BMHSCs for transplantation into recipient mice, the cells were cultured with the lentiviruses for 2 days before injection into recipient mice. Four groups of four 8 week-old Balb/c male mice each were lethally irradiated with a single dose of 800 cGy of γ -irradiation. Four hours after irradiation, 2 $\times$ 10⁶ transduced BMHSCs were injected intravenously into each recipient mouse in 200 μ l sterile PBS. To reduce infection, the mice were given acidified water for drinking (0.01% HCl) one week before irradiation/transplantation and for 4 weeks post transplantation.

Flow cytometric analysis

For analysis of EGFP transgene expression, transduced cell lines were harvested, washed in PBS (pH 7.2) and re-suspended in PBS containing 2% FCS. The cells were analysed on a FACSCalibur using CellQuest software (BD Biosciences). Successful culture/differentiation

of BMHSCs *in vitro*, reconstitution *in vivo* and expression of EGFP in primary T- and B-lymphocytes in the blood and splenocytes of recipient mice was determined by two colour FACS following staining with PE-conjugated anti-CD19 for B-lymphocytes or PE anti-CD4 for T-lymphocytes. Blood from recipient mice was collected after 2 and 3 months and spleens after 3 months post transplantation. Blood was collected from the tail vein in heparinized glass capillary tubes, erythrocytes were lysed with the Red Blood Cell lysis buffer (Sigma-Aldrich).

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Figure 1.

A schematic diagram illustrating the structure of lentiviral vectors used in the study. The SFFV-EGFP vector encodes enhanced green fluorescence protein (EGFP) from the spleen focus-forming virus (SFFV) promoter. The E μ -MAR-PGK-EGFP lentiviral vector encodes EGFP from phosphoglycerate kinase (PGK) promoter. The engineered CD19-EGFP and E μ -MAR-CD19-EGFP lentiviral vectors both encode EGFP from the CD19 promoter. The E μ flanked with matrix attachment regions (MAR) were incorporated upstream of the PGK and CD19 promoter. Viruses were produced by co-transfecting HEK-293T cells with the vectors together with the pCMV Δ R8.2 packaging plasmid and pMD.G encoding the VSVG envelope. Abbreviations: Ψ , Psi domain; LTR, long terminal repeat; RRE, rev-responsive element; WPRE, woodchuck posttranscriptional regulatory element.

Figure 2.

Flow cytometric profiles of EGFP protein expression by a B-lymphocyte cell line (Ramos), a T-lymphocyte line (Jurkat) and a fibroblast cell line (HEK-293T) transduced with the lentiviral vectors SFFV-EGFP or Eμ-MAR-PGK-EGFP. Percent cells expressing EGFP were determined (indicated) by expression of green fluorescence and by side scatter (SSC).

Figure 3.

Flow cytometric profiles of EGFP expression. The profiles depict percentages of human and murine B-cell lines (Ramos and LK-35) and T cells (Jurkat and BW5147) expressing EGFP after transduction with the SFFV-EGFP, CD19-EGFP or Eμ-MAR-CD19-EGFP lentiviral vectors. The results presented in the figure show data from one experiment. The experiment was repeated at least three times on different days and using different batches of lentiviruses and cells. The SFFV-EGFP vector induced EGFP expression in $21.7 \pm 2.9\%$ (mean $\pm$ s.e.m.) Ramos B cells, $20.0 \pm 1.5\%$ LK-35 B cells, $58.3 \pm 7.3\%$ Jurkat T cells and $56.7 \pm 4.9\%$ BW5147 murine T-cell line. The CD19-EGFP induced EGFP expression in $16.7 \pm 3.4\%$ Ramos B cells, $11.0 \pm 1.5\%$ LK-35 B cells, $2.3 \pm 0.3\%$ Jurkat T cells and $1.3 \pm 0.3\%$ BW5147 cells. The Eμ-MAR-CD19-EGFP vector induced EGFP expression in $18.3 \pm 1.8\%$ Ramos cells, $21.7 \pm 2.2\%$ LK-35 cells, $3.3 \pm 0.7\%$ Jurkat cells and $4.3 \pm 0.3\%$ BW5147 cells. The Eμ-MAR elements enhanced the level of EGFP expression (mean fluorescence intensity; MFI) by 3–5 folds in the B-cell lines.

Figure 4.

Immune reconstitution of lethally irradiated mice with transduced BMHSCs. Two groups of four, 8-week-old Balb/c mice each were studied. One group was irradiated with 800 cGy of γ -irradiation but not reconstituted with BMHSCs and used as control to determine the effectiveness of the irradiation. The second group was irradiated and transplanted intravenously with 2×10^6 BMHSCs from 8-week-old Balb/c mice from the same colony. FACS analysis of peripheral blood was performed on both groups to determine numbers of B- and CD4⁺ T-lymphocytes before and after transplantation using FITC-conjugated anti-CD19 or anti-CD4 antibodies. (a) The proportion of B- and T-lymphocytes before irradiation are shown, (b) 1 week after irradiation of the control group, (c) 10 weeks after transplantation with BMHSCs. Before irradiation, $49.8 \pm 0.9\%$ of blood nucleated cells within the lymphocyte gate were CD4⁺ T-lymphocytes and $22.3 \pm 0.5\%$ were CD19⁺ B-lymphocytes. After irradiation and reconstitution of the second group with BMHSCs, the distribution of lymphocytes within the lymphocyte gate were $51.0 \pm 2.2\%$ CD4⁺ T-lymphocytes and $22.8 \pm 1.4\%$ CD19⁺ B-lymphocytes.

Figure 5.

Expression of EGFP in lymphocytes in mice transplanted with lentiviral transduced BMHSCs. Mean and s.e.m. for FACS data on the analysis of lymphocytes expressing EGFP: (a) histograms representing per cent B-lymphocytes (grey columns) and non-B-lymphocytes (black columns) within the lymphocyte gates that expressed EGFP in the blood (left) and spleen (right) of recipient mice. (b) Per cent B- and non-B-lymphocytes within the EGFP-positive gate. (c) MFI of EGFP expression levels in B- and non-B-lymphocytes in the blood and spleen of mice recipients of HBMSCs transduced with lentiviral vectors SFFV-EGFP, CD19-EGFP or Eμ-MAR-CD19-EGFP.