Transgenic Res (2010) 19:105–112
DOI 10.1007/s11248-009-9288-6

BRIEF COMMUNICATION

Characterisation of eGFP-transgenic BALB/c mouse strain established by lentiviral transgenesis

Krisztián Kvell · Tamás Czömpöly · László Hiripi · Péter Balogh · József Kóbor · Lilla Bodrogi · Judit E. Pongrácz · William A. Ritchie · Zsuzsanna Bősze

Received: 23 February 2009 / Accepted: 19 May 2009 / Published online: 31 May 2009
© Springer Science+Business Media B.V. 2009

Abstract Lentiviral technology is a powerful tool for the creation of stable transgenic animals. However, uncertainties have remained whether constitutive promoters resist long-term silencing. We used concentrated HIV-1 based lentiviral vectors to create stable transgenic BALB/c mice by perivitelline injection. In our vectors eGFP expression was driven by the human EF1α promoter. The established transgenic animals were analyzed for eGFP expression by in vivo fluorescence imaging, PCR, histology and flow-cytometry. eGFP expression showed even distribution without mosaicism; however, tissue-dependent differences of eGFP expression were observed. Up to the sixth generation only one newborn showed eGFP inactivation. eGFP + transgenic bone marrow cells efficiently provided long-term haemopoietic repopulation in radiation chimeras, regenerating all bone marrow-derived lineages with eGFP + cells with distinct eGFP expression profiles. The established eGFP + BALB/c mouse strain is expected to be extremely useful in various immunological experiments.

Keywords Balb/c · eGFP · Lentiviral transgenesis · Bone-marrow chimeric mouse

K. Kvell · T. Czömpöly · P. Balogh · J. E. Pongrácz
Department of Immunology and Biotechnology, University of Pecs, Pecs, Hungary

L. Hiripi · L. Bodrogi · Z. Bősze (✉)
Genetic Modification Program Group, Agricultural Biotechnology Center, P. O. Box 411, 2100 Gödöllő, Hungary
e-mail: bosze@abc.hu

J. Kóbor
Department of Oncotherapy, University of Pecs, Pecs, Hungary

W. A. Ritchie
Roslin Embryology Limited, Edinburgh, Scotland, UK

Introduction

Lentiviral transgenesis has become an important and efficient new tool for the establishment of transgenic animals. The transgenic sequence most often used for 'proof-of-principle'-type experiments is eGFP (enhanced green fluorescent protein). Concentrated lentiviral vectors efficiently transfect zygotes following perivitelline injection. With this method the nucleus of the zygote is not affected directly, and higher volumes may be injected with less sophisticated machinery. With lentiviral vectors the efficiency of stable, active transgenesis is significantly higher than with standard pronuclear injection, reaching 8–50% (Park 2007).

In the field of immunology a massive body of experimental evidence is derived from studies performed in mice with BALB/c genetic background (Bleul et al. 2006; Ivanov et al. 2006; Odegaard et al.

2007; Suzuki et al. 2006; Tezuka et al. 2007; Wing et al. 2008). However, BALB/c mice are difficult to manipulate by pronuclear injection, unlike the FVB/n strain which is characterized by vigorous reproductive performance, consistently large litters and prominent pronuclei facilitating the microinjection of DNA (Taketo et al. 1991). Consequently a widely used approach is to generate genetically modified mice on a genetic background which facilitates micromanipulation, and backcross the offsprings to BALB/c background for at least ten generations. To address this issue we sought to generate eGFP transgenic mice directly on BALB/c background by lentiviral transgenesis. Though eGFP is considered as a functionally inert intracellular protein with minimal tendency to interfere with physiological processes both in vitro and in vivo, we wished to examine in our system whether the presence of eGFP in the BALB/c mouse strain interferes with immunological processes in either central or peripheral immune organs. To investigate integration stability and transgene activity following lentiviral transgenesis as well as possible eGFP-triggered interference with immunological processes we also performed long-term in vivo experiments involving haemopoietic stem cell (HSC) transfer.

Results and discussion

Efficiency and transgene inheritance following lentiviral transduction

Purified, concentrated lentiviral supernatant was used for perivitelline injection in both BALB/c and FVB/n mice. Toxicity of viral supernatant was low as high percentages of injected and transferred embryos were born with both mouse strains (60% or 12/20 with BALB/c vs. 66% or 27/41 with FVB/n). Visual observation of newborns for identifying eGFP + transgenic BALB/c mice revealed uniform eGFP expression, lacking traits of mosaicism (Fig. 1a). The presence of integrated transgenic sequence was verified by eGFP PCR performed on genomic DNA and yielded similar efficiencies with both mouse strains among newborn animals (58% or 7/12 with BALB/c vs. 48% or 13/27 with FVB/n). The overall efficiency of active transgenesis (ratio of visually eGFP positive animals and initially injected zygotes) was equally high in both mouse strains examined (20% or 4/20 with BALB/c vs. 22% or 9/41 with FVB/n). An eGFP expressing male BALB/c founder was used to establish the transgenic line through mating with wild type BALB/c females. Southern analysis (Fig. 1b) and the ratio of transgenic animals in the F1 litters indicated one transgene integration site.

The presence of eGFP as a transgenic protein did not abrogate either the macroscopic development of transgenic BALB/c mice or their reproductive abilities; litter sizes were similar to control BALB/c mice (6–7 pups). Long-term transgene stability was remarkable as up to six generations only one single offspring showed loss of detectable eGFP fluorescence. (It has not been tested whether transgene loss or inactivation was responsible for this event). Calculating with 6 pups as average litter size this means that the loss of transgene/transgene activity has occurred at a very low frequency at approx. 3% (1/36). This solid heredity may partly be due to the rigorous flow-cytometric screening performed from blood samples of the F1 generation members, following which only those transgenic animals were further bred that showed unambiguously uniform eGFP expression in peripheral blood mononuclear cells (data not shown). Such stability of transgene inheritance and activity is especially impressive with the notion of utilizing a short, constitutively active promoter (human EF1α). Our results on stable transgene inheritance line up with earlier data obtained with EF1α-eGFP transgenic rats obtained by the lentiviral transduction of spermatogonial stem cells (Ryu et al. 2007).

Analysis of transgene expression in eGFP + BALB/c mice

Tissue samples of eGFP + BALB/c mice were analyzed from several samples by histology and flow-cytometry. Figure 1c–f summarizes representative histological results of cryostat sections of muscle, intestine, spleen and lymph node. eGFP expression is present within the cytoplasm of all nucleated cells. There were significant differences of fluorescence intensity among various tissue and cell types. In lymphoid organs, the mesenchymal-reticular cells and vasculature appeared to express eGFP more heavily than the surrounding lymphocytes. This phenomenon may be the consequence of the promoter sequence used. Although EF1α is ubiquitously active non-viral

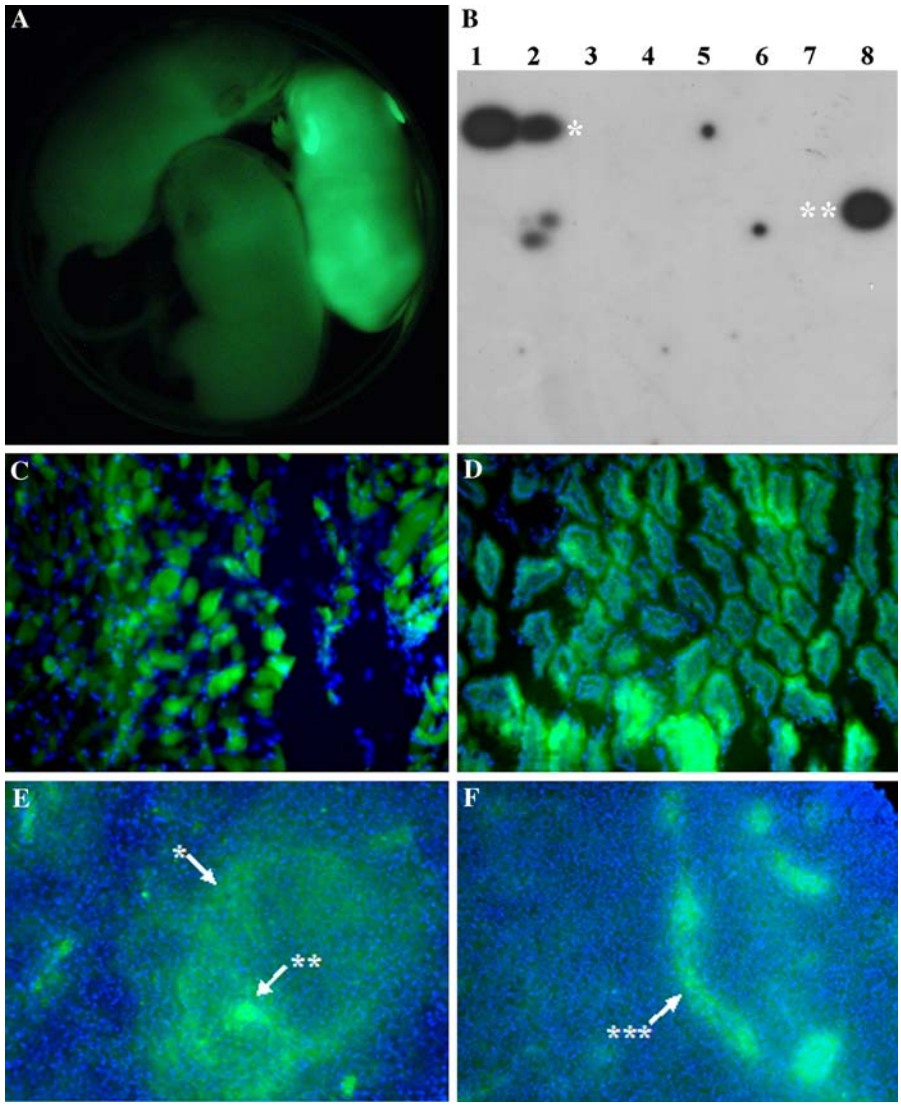

Fig. 1 Macroscopic identification, Southern and histological analysis of eGFP + BALB/c mice. **a** Shows an eGFP + newborn BALB/c mouse (*top right*) under fluorescent light among control littermates. **b** Shows Southern analysis to determine integrated transgene copy number. *Lanes 1 and 2*: eGFP + Balb/c mice. *Lanes 3, 4, and 5*: non-transgenic Balb/c mice. *Lanes 6 and 7*: no DNA added and negative plasmid DNA, *lane 8*: the LTR-cPPT-EF1α-GFP-WPRE-LTR transfer vector. Fragment sizes * >10 kb; ** 7.0 kb. **c–f** show eGFP expression in cryostat sections of muscle (**c**), intestine (**d**), spleen (**e**) and lymph node (**f**) counterstained with DAPI. Intense eGFP fluorescence is associated with stromal-fibroblastic scaffolding of white pulp (*) and vascular elements, including splenic central arteriole (**) and high endothelial venule in lymph node (***)

promoter, there may be significant differences for activity depending on the actual context of gene expression. Alternatively, cells with larger cytoplasmic volume may contain more eGFP molecules, rendering these cells more fluorescent, similar to differences observed during flow-cytometric analyses below.

Figure 2 summarizes the results of flow-cytometric characterization of peripheral blood, thymus and lymph node. The difference of eGFP fluorescence intensity was also obvious by the flow-cytometric analysis of peripheral blood leukocytes. Figure 2a shows peripheral blood leukocytes distinguished by morphology following the lysis of erythrocytes (left figure of Fig. 2a). Three distinct peaks of eGFP fluorescence intensity are seen on the FL1 histogram (middle figure of Fig. 2a), defined by gates R1 (low-), R2 (mid-) and R3 (high intensity). Following backward morphological representation using these gates (right figure of Fig. 2a) cells of weak fluorescence (R1 gate, light gray), medium fluorescence (R2 gate, medium gray) and high fluorescence (R3 gate, high fluorescence) are identified. It appeared that eGFP fluorescence strongly correlated with cell size (FSC scatter) in peripheral blood leukocytes. In the thymus, surface staining was performed to differentiate developing thymocyte subpopulations. Similarly, mature lymphocytes in the lymph node were identified by

CD4/CD8 double staining (Fig. 2b–e), and compared to wild-type BALB/c controls. Mosaicism was not observed by either histology or flow-cytometry. Our results show near identical distribution profiles of developing thymocytes in the thymus and mature lymphocytes in the lymph node. Recent data shown preferential expression of the GFP in the exocrine cells of transgenic pigs, where the GFP transgene was placed under the strong cytomegalovirus (CMV) enhancer-promoter (Vasey et al. 2009).

Analysis of eGFP expression in bone-marrow (BM) chimeric BALB/c mice

To determine whether eGFP-expressing transgenic BALB/c mice are suitable donors for haemopoietic reconstitution experiments, wild-type BALB/c mice were irradiated and repopulated with bone marrow cells from eGFP + BALB/c donors. The degree of chimerism and eGFP-expression pattern was analyzed 6 weeks after bone marrow transplantation. Figure 3 summarizes the results of flow-cytometric characterization of bone marrow, peripheral blood and lymph node samples from BM-chimeric mice. Histogram plot of Fig. 3b shows the efficacy of eGFP + HSCs for reconstituting the haemopoietic system (over 90% of BM cells fall positive in gate M1). Mosaicism among peripheral blood leukocytes was not detected following haemopoietic reconstitution. We also determined the ratio of different peripheral blood leukocytes by flow-cytometry following the lysis of erythrocytes (see Fig. 3c and d). Region statistics of wild-type and eGFP-chimeric BALB/c mice show similar distribution pattern and ratio of major leukocyte groups differentiated by morphological representation. Surface staining for CD4/CD8 antigens was performed to differentiate mature lymphocytes in the lymph node of control and BM chimeric BALB/c mice. Our results show indistinguishable distribution profiles of mature lymphocytes in the lymph nodes of control and BM chimeric BALB/c mice (see Fig. 3e and f). These data indicate that the presence of eGFP in BALB/c HSCs does not impair their capacity to restore haemopoiesis.

In summary, lentiviral transgenesis is a powerful tool for the creation of stable transgenic lines with mouse strains that are challenging for standard pronuclear injection, like the BALB/c in our case. Major immunological parameters of the established eGFP + BALB/c mouse strain were elaborately characterized and found to be equal to that of control BALB/c mice. In the future we plan to use this transgenic strain and take advantage of marker gene-harboring murine cells to investigate i.e. tumor formation, tumor-cell dissemination, lymphocyte homing and recirculation and several other applications.

Materials and methods

Plasmid constructs

Late second generation lentiviral sequences are divided into three plasmid constructs. The envelope construct pMD.G encodes the viral envelope protein pseudotyped with VSV-G (vesicular stomatitis virus G-protein). The packaging construct R8.91 encodes enzymatic and structural elements required for the assembly of recombinant retroviral vectors. The transfer construct pWPTS carries the transgene of interest in an LTR (long terminal repeat) frame. This construct also has cPPT (central polypurine tract) for increased nuclear transport, WPRE (Woodchuck-hepatitis virus post-transcriptional responsive element) for prolonged mRNA half-life and SIN (self-inactivating) mutation in the LTR frame. The HIV-1 derived lentiviral system was kindly provided by Prof. Didier Trono (CMU, Geneva, Switzerland).

Lentivirus production, purification, concentration, titration

Lentiviral particles were produced as described previously (Bovia et al. 2003; Kvell et al. 2005). Briefly, the three plasmid constructs were co-transfected into 293T cells using the calcium–phosphate method. Viral supernatant was harvested after incubation for 48 h at 37°C, 5% CO_2. Purification steps included centrifugation (2,000 rpm, 10 min, 4°C) and filtration (PVDF-coated 0.45 μm filters). For concentration the supernatant was layered on 20% sucrose for ultra-centrifugation (26,000 rpm, 90 min, 4°C). Viral particles were re-suspended in DMEM. Biological titration was performed with HeLa cells. Viral particles were concentrated 1,000-fold in volume; biological titers reached and exceeded 10^8 TU/ml.

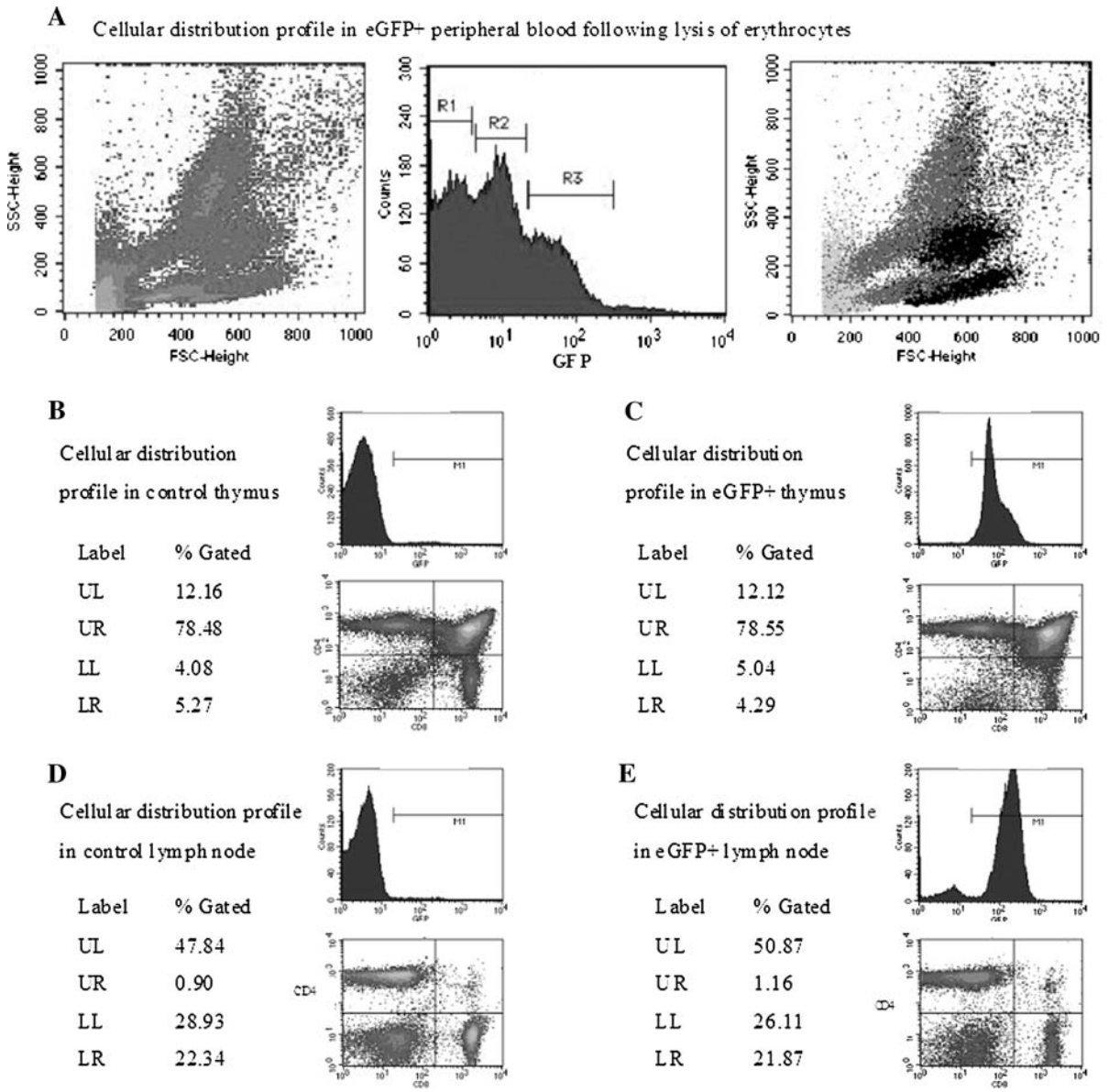

Fig. 2 Flow-cytometric characterization of eGFP + BALB/c mice. **a** Shows cellular distribution profile of eGFP fluorescence intensity in peripheral blood following erythrocyte lysis. *Left* density plot figure shows cell groups distinguished by morphology. *Middle* figure shows eGFP fluorescence intensity distribution profile as FL1 fluorescence histogram. Three distinct cell groups are differentiated by fluorescence intensity defined by gates R1 (low-), R2 (mid-) and R3 (high intensity). Dot plot figure on the *right* shows backward morphological representation. This identifies cells falling into gates R1 (*light gray*), R2 (*mid-gray*) and R3 (*dark gray*). **b** and **c** Show the cellular distribution profile (density plot) of thymocyte subgroups in thymi of control and eGFP + BALB/c mice, respectively. **d** and **e** Show the cellular distribution profile (density plot) of lymphocyte subgroups in lymph node control and eGFP + BALB/c mice, respectively. For comparison, quadrant statistics are enclosed to the figures

Zygote isolation, perivitelline injection, implantation

All strains (control and transgenic) of BALB/c and FVB/n mice were kept in conventional animal house. Lentiviral vector injection was performed based on the modified perivitelline space injection method to produce transgenic mice from low titer lentiviral vector (Ritchie et al. 2007). Approximately 300–500 pl of low titer (10^6–10^8 infectious units per ml)

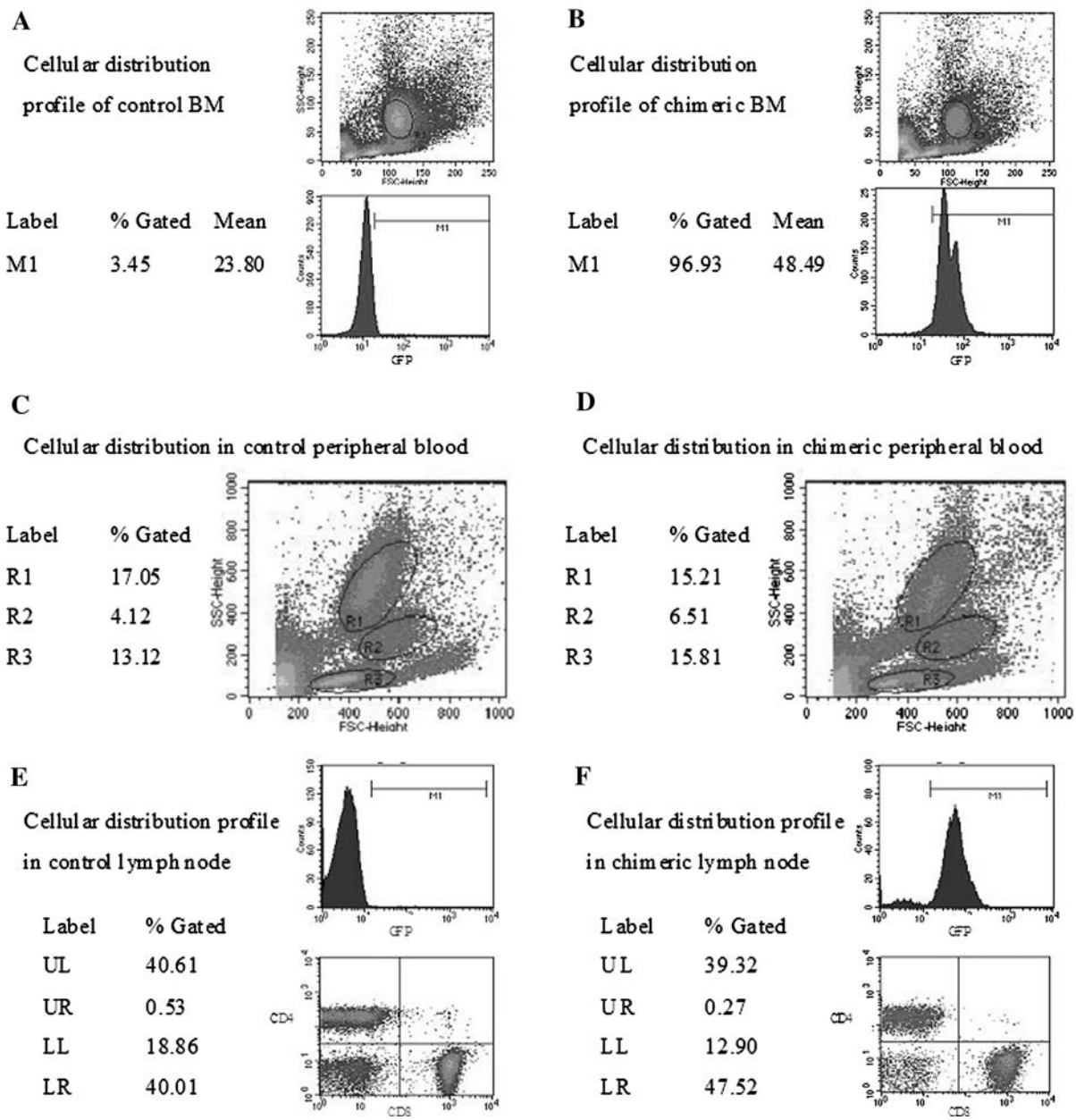

Fig. 3 Flow-cytometric characterization of BM chimeric BALB/c mice. **a** and **b** Show the cellular distribution profile (morphological and histogram plots) of bone marrow (BM) cells of control and BM chimeric BALB/c mice, respectively. For comparison, histogram statistics are shown. **c** and **d** Show cellular distribution (density plots) in peripheral blood from control and BM chimeric animal, respectively. For comparison, region statistics are shown. **e** and **f** Show the cellular distribution profile (histogram and density plots) of lymph node lymphocytes of control and BM chimeric BALB/c mice, respectively. For comparison, quadrant statistics are enclosed to both figures

lentiviral vector was repeatedly injected into the perivitelline space of single cell embryo. Superovulation of donor females, embryo isolation and transfer into surrogate mothers was performed according to standard laboratory protocols.

Bone-marrow chimeric mice were provided with ciprofloxacin during the initial 4 weeks following bone marrow transplantation.

Experiments were approved by the Animal Care and Ethics Committee of the Agricultural Biotechnology

Center and complied with the Hungarian Code of Practice for the Care and Use of Animals for Scientific Purposes, including conditions for animal welfare and handling prior to slaughter.

In vivo fluorescence imaging

eGFP and auto-fluorescence was assayed using blue light illumination (GFP excitation frequency 455–495 nm with a barrier filter cut of below 500 nm). For newborn and adult mice this was performed with a GFSP-5 headset (Biological Laboratory Instruments, Budapest).

Detection of transgene integration

Lentiviral transgene integration number was determined by Southern blot analysis of DNA from tail biopsy. 20 μg of DNA was digested with BamHI, separated on a 1% agarose gel, blotted to a nylon membrane and probed with a 282 bp-long PCR amplified eGFP fragment using primers GFP-forward 5′-CTCGTGACCACCCTGACCTAC-3′ and GFP-reverse 5′-CATGATATAGACGTTGTGGCTGTT-3′.

Detection of eGFP by histology

10 μm thick cryostat sections were prepared from various snap frozen tissues of eGFP + BALB/c mice, including muscle, intestine, spleen and lymph node, and fixed in 4% buffered paraformaldehyde to preserve native eGFP fluorescence. Nuclei were counterstained with DAPI. The specimens were viewed under an Olympus BX61 fluorescent microscope. The digital pictures were acquired with a CCD camera using the analySIS® software.

Detection of eGFP by flow-cytometry

Cells were obtained from bone marrow, thymus, lymph node and blood and peripheral blood. The lymphoid tissues were mechanically dissociated, single cell suspensions were prepared and were stained for CD4 and CD8 (BD, clones: H129.9 (a-CD4) and 53-6.7 (a-CD8)) in PBS containing 0.1% BSA and Na-azide). Stained cells were fixed with paraformaldehyde, measured by FACSCalibur flow-cytometer and analyzed by CellQuest Pro (Becton Dickinson). Cell populations were differentiated by morphology gates.

Protocol of bone marrow transplantation

About 6–8 weeks old BALB/c mice were irradiated with 10 Gy in a single bolus calculated at midline level using a Co^{60}-source. 6 h later irradiated mice were injected intravenously with 200 μl cell suspension of bone marrow cells isolated from eGFP + BALB/c adjusted to 10^7 viable cells/ml. Six weeks after haemopoietic reconstitution cells were obtained, stained and measured as above.

Acknowledgments This work was supported by OTKA T 049034 and OMFB-00118/2008 to BZS, OTKA PD 78310 to KK. TC is a recipient of the Bolyai János Postdoctoral Fellowship of the Hungarian Academy of Sciences. The authors thank G. Takács for the artwork.

References

Bleul CC, Corbeaux T, Reuter A, Fisch P, Monting JS, Boehm T (2006) Formation of a functional thymus initiated by a postnatal epithelial progenitor cell. Nature 441:992–996. doi:10.1038/nature04850

Bovia F, Salmon P, Matthes T, Kvell K, Nguyen TH, Werner-Favre C, Barnet M, Nagy M, Leuba F, Arrighi JF, Piguet V, Trono D, Zubler RH (2003) Efficient transduction of primary human B lymphocytes and nondividing myeloma B cells with HIV-1-derived lentiviral vectors. Blood 101:1727–1733. doi:10.1182/blood-2001-12-0249

Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, Littman DR (2006) The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17 + T helper cells. Cell 126:1121–1133. doi:10.1016/j.cell.2006.07.035

Kvell K, Nguyen TH, Salmon P, Glauser F, Werner-Favre C, Barnet M, Schneider P, Trono D, Zubler RH (2005) Transduction of CpG DNA-stimulated primary human B cells with bicistronic lentivectors. Mol Ther 12:892–899. doi:10.1016/j.ymthe.2005.05.010

Odegaard JI, Ricardo-Gonzalez RR, Goforth MH, Morel CR, Subramanian V, Mukundan L, Eagle AR, Vats D, Brombacher F, Ferrante AW, Chawla A (2007) Macrophage-specific PPARgamma controls alternative activation and improves insulin resistance. Nature 447:1116–1120. doi:10.1038/nature05894

Park F (2007) Lentiviral vectors: are they the future of animal transgenesis? Physiol Genomics 31:159–173. doi:10.1152/physiolgenomics.00069.2007

Ritchie WA, Neil C, King T, Whitelaw CB (2007) Transgenic embryos and mice produced from low titre lentiviral vectors. Transgenic Res 16:661–664. doi:10.1007/s11248-007-9102-2

Ryu BY, Orwig KE, Oatley JM, Lin CC, Chang LJ, Avarbock MR, Brinster RL (2007) Efficient generation of transgenic rats through the male germline using lentiviral transduction and transplantation of spermatogonial stem cells. J Androl 28:353–360. doi:10.2164/jandrol.106.001511

Suzuki N, Suzuki S, Millar DG, Unno M, Hara H, Calzascia T, Yamasaki S, Yokosuka T, Chen NJ, Elford AR, Suzuki J, Takeuchi A, Mirtsos C, Bouchard D, Ohashi PS, Yeh WC, Saito T (2006) A critical role for the innate immune signaling molecule IRAK-4 in T cell activation. Science 311:1927–1932. doi:10.1126/science.1124256

Taketo M, Schroeder AC, Mobraaten LE, Gunning KB, Hanten G, Fox RR, Roderick TH, Stewart CL, Lilly F, Hansen CT et al (1991) FVB/N: an inbred mouse strain preferable for transgenic analyses. Proc Natl Acad Sci USA 88:2065–2069. doi:10.1073/pnas.88.6.2065

Tezuka H, Abe Y, Iwata M, Takeuchi H, Ishikawa H, Matsushita M, Shiohara T, Akira S, Ohteki T (2007) Regulation of IgA production by naturally occurring TNF/iNOS-producing dendritic cells. Nature 448:929–933. doi:10.1038/nature06033

Vasey DB, Lillico SG, Sang HM, King TJ, Whitelaw CB (2009) CMV enhancer-promoter is preferentially active in exocrine cells in vivo. Transgenic Res 18:309–314. doi:10.1007/s11248-008-9235-y

Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, Nomura T, Sakaguchi S (2008) CTLA-4 control over Foxp3 + regulatory T cell function. Science 322:271–275. doi:10.1126/science.1160062