Cell-Specific Transduction of *Prdm1*-Expressing Lineages Mediated by a Receptor for Avian Leukosis Virus Subgroup B[†]

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The transcription factor Blimp-1 has emerged as a regulator of cell fate in embryonic (germ cell) and adult (B- and T-cell immune effector and epithelial) lineages. It has also been proposed to act as a tumor suppressor in B-cell malignancy. Here, we present a novel in vivo system enabling the targeted genetic manipulation of cells expressing *Prdm1*, the gene encoding Blimp-1. We created bacterial artificial chromosome-transgenic mice expressing the avian leukosis virus (ALV) receptor TVB, fused to monomeric red fluorescent protein, under regulation by *Prdm1* transcriptional elements, and we achieved transduction of TVB-expressing lymphocytes by ALV vectors bearing a subgroup B envelope. The system presented here incorporates a number of innovations. First, it is the first mammalian transgenic system that employs the ALV receptor TVB, thus expanding the flexibility and scope of ALV-mediated gene delivery. Second, it represents the first ALV-based system that allows gene transfer and expression into in vivo-activated mature lymphocytes, a cell type that has traditionally presented formidable challenges to efficient retroviral transduction. Third, *Prdm1*:TVB-mRFP transgenic animals could provide an invaluable tool for exploring the diverse roles of Blimp-1 in lineage commitment, immune regulation, and tumorigenesis.

The development of in vivo models that enable the genetic manipulation of cells in the earliest stages of commitment to a specific lineage could provide a powerful means for studying normal development as well as recreating the clonal origins of cancer experimentally.

Tumors derived from mature, antigen-experienced lymphocytes often express *Prdm1*, the gene encoding the transcription factor Blimp-1. Blimp-1 is a zinc finger-containing transcriptional regulator with key roles in cell fate specification in diverse embryonic and adult lineages (29). During mouse embryogenesis, Blimp-1 commits primordial cells to a germ cell fate (32). In the adult, Blimp-1 is indispensable for the maturation and/or function of important subsets of immune effector cells as well as some epithelial cells, including subsets with stem cell properties (18, 28).

Blimp-1 was initially identified as the master regulator of plasma cell differentiation (2, 19, 22, 26, 27, 37, 40–42, 45). Conditional deletion of *Prdm1* in the B-cell lineage results in abrogation of plasma cell development (41). Mature plasma cells, resident in the bone marrow, remain dependent on continued expression of *Prdm1* (42). Malignant plasma cells in multiple myeloma, as well as malignant B cells in some cases of mature B-cell lymphomas, also express *Prdm1* (6). In other cases, inactivation of *Prdm1* by mutation, deletion, or transcriptional silencing has been proposed to prevent differentiation of malignant B cells, thus attributing a tumor suppressor role to *Prdm1* in diffuse large B-cell lymphoma (36, 44). More recently, Blimp-1 has been credited with critical functions in

the maturation and homeostasis of effector T cells (12, 23, 29–31, 38), including regulatory T cells (48). Given the central role of this transcriptional regulator in development, immunity, and cancer, we developed a transgenic system designed to manipulate *Prdm1*-expressing lineages genetically in a flexible, somatic, tissue-specific fashion.

In order to create such a system, we ectopically expressed an allele of the avian leukosis virus (ALV) receptor locus *Tv-b*, *Tv-b*^{S3}, under transcriptional regulation by elements belonging to the *Prdm1* locus. Mammalian cells are normally refractory to infection by avian retroviruses, unless they are engineered to express a cognate avian-derived retroviral receptor (3, 11, 20, 46, 47). Ectopic expression of an ALV receptor by a mammalian cell confers susceptibility to infection by a viral vector bearing the appropriate avian-derived envelope glycoprotein. To date, only one of the loci encoding ALV receptors, *Tv-a*, has been used for transgenic experiments (8, 9, 13, 15, 16, 25, 33–35, 39). *Tv-a* encodes the receptor for subgroup A-ALV, TVA. Various alleles of a second ALV receptor locus, *Tv-b*, encode receptors for subgroup B, D, and E viruses (1, 7, 43). The allele *Tv-b*^{S3} mediates transduction by subgroup B and D ALV. The protein product of *Tv-b*^{S3}, TVB^{S3}, is related to mammalian death receptors (type I tumor necrosis factor receptor and Fas) and, in its native form, incorporates a death domain in the intracellular portion (4). A version of TVB^{S3} lacking the intracytoplasmic “death domain” [TVB^{S3}-ΔDD(4)] was used in experiments described here to reduce the probability that the transgene would interfere with normal development or cell function of the transgenic mice. For simplicity, the product of the truncated *Tv-b*^{S3} allele will be referred to as “TVB” throughout the rest of this paper.

MATERIALS AND METHODS

BAC clones. The bacterial artificial chromosome (BAC) clone RP23-217C20 was obtained from the Children’s Hospital of Oakland Research Institute repos-

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itory (<http://www.chori.org/>). BAC-containing bacterial stocks were propagated in LB medium supplemented with chloramphenicol.

BAC modification by homologous recombination in *Escherichia coli*, transgene preparation, and transgenic animal generation. Plasmid pJJ2, encoding TVB^{S3} minus the native cytoplasmic death domain [TVB^{S3}- Δ DD(4)], was a kind gift from Juergen Brojatsch, Albert Einstein Medical School. A plasmid encoding monomeric red fluorescent protein (mRFP1) was a kind gift from Roger Tsien, UCSD. Using a PCR method (see the supplemental material), mRFP was fused in-frame to the sequences encoding TVB^{S3}- Δ DD and the resultant fragment placed upstream of an simian virus 40 poly(A) signal. Further rounds of PCR were used to insert 5' and 3' homology boxes at the flanks of the TVB-mRFP-poly(A) fragment (for explanation, see the supplemental material). The 5' homology box corresponds to the region immediately preceding the Blimp-1 translation initiation codon, ATG, in the *Prdm1* locus. The 3' homology box corresponds to a region downstream of the aforementioned ATG codon. The primer sequences are appended in supplemental material.

The resultant TVB-mRFP fusion fragment, followed by a simian virus 40 poly(A) site and flanked by *Prdm1*-derived 5' and 3' homology boxes (as described above), was cloned into the *AscI*/*FseI* sites of shuttle vector pLD53.SC-AB, obtained from Nathaniel Heintz, Rockefeller University. The complete pLD53.SC-AB:TVB-mRFP shuttle vector was electroporated into *E. coli* harboring BAC RP23-217C20. Selection for recombinants was carried out as detailed elsewhere (14). Southern blotting was used to screen for recombinants at each step (see Fig. S1 and S2 in the supplemental material). At the conclusion of the process, *SapI* digestion of parental and modified BAC DNA was used to exclude gross rearrangements (see Fig. S2 in the supplemental material). BAC DNA was purified using a Qiagen large construct kit (Qiagen). BAC DNA was microinjected into mixed-background, fertilized oocytes (FVB/NJ crossed with C57BL/6J). Manipulated oocytes were reimplanted into the uteri of pseudopregnant female mice and the resulting embryos carried to term per the standard procedure. Four founders (two male and two female) were identified among 25 progeny; all four founders transmitted the transgene in the germ line. One line, BBAC13, was used for subsequent experiments. The transgene was backcrossed into a pure C57BL/6J background for 10 generations. All animals were housed in the MSKCC Animal Facility and cared for according to institutional guidelines under an approved IACUC animal care protocol (05-11-025).

Immunization. Adolescent animals (6 to 8 weeks old) were immunized with an intraperitoneal injection of 100 μ l of a 10% suspension of washed sheep red blood cells (Rockland Immunochemicals). Two to three weeks after the primary immunization, an identical booster immunization was administered. Some animals received subsequent booster immunizations at 2-week intervals.

FACS analysis. After red blood cell lysis, splenocyte suspensions were incubated with mouse Fc block (BD Biosciences) and primary antibodies (biotin anti-CD138, biotin anti-B220, biotin anti-Ter119, biotin anti-Gr-1, biotin anti-DX-5, biotin anti-CD25, and biotin anti-CD8), followed by a brief incubation with streptavidin-fluorescein isothiocyanate (FITC) (all reagents were obtained from BD Biosciences). FITC-conjugated anti-CD4 and FITC-conjugated anti-CD90.2 were obtained from BD Biosciences. Stained samples were analyzed by fluorescence-activated cell sorting (FACS) analysis using a MoFlo analyzer (Dako Cytomation) with a 568-nm laser.

Immunocytochemistry. Frozen sections were air dried, fixed in methanol and then cold acetone (5 min each), and incubated with the following primary antibodies: anti-red fluorescent protein rabbit polyclonal antibody (dilution, 1:200; Rockland Immunochemicals), anti-Blimp-1 rat monoclonal antibody (clone 6D3; dilution, 1:50; Santa Cruz Biotechnology), and anti-Ki67 rabbit polyclonal antibody (dilution, 1:200; Novocastra). Biotinylated secondary anti-rat immunoglobulin G/anti-rabbit immunoglobulin G F(ab')₂ fragments were obtained from Fitzgerald Industries. For immunocytochemistry, the Vector Laboratories avidin/biotin (ABC) system was used. Peroxidase substrate (DAB [3,3'-diaminobenzidine tetrahydrochloride]) and alkaline phosphatase substrate (BCIP [5-bromo-4-chloro-3-indolylphosphate]-nitroblue tetrazolium [NBT]) were used as chromogens.

ALV vector propagation and preparation of high-titer virus stocks. Chicken embryonic fibroblasts (DF-1 cell line; ATCC) were transfected with RCAS or RCAN ALV vector plasmids by using liposome-mediated transfection (Fugene 6; Roche). Transfected producer cells were cultured for approximately 2 weeks to allow viral propagation throughout the culture. Supernatant from six confluent 150-mm plates was pooled and filtered through a 0.45- μ m filter to collect dead cells and debris. The pooled supernatant was then carefully layered over a cushion of 20% sucrose (5 ml at the bottom of a 36-ml polyallomer tube). Tubes were then ultracentrifuged at a speed of 23,000 rpm at 4°C for 1 h in a Sorvall ultracentrifuge. The supernatant was removed by aspiration, leaving 200 to 500 μ l overlying the (invisible at this stage) viral pellet. This was allowed to resus-

pend by gentle rocking at 4°C for 1 h. The resuspended pellets were combined and brought to a final volume of 10 ml with sterile, cold phosphate-buffered saline. The combined suspension was then centrifuged for a second time at 23,000 rpm at 4°C for 1 h. Following the second centrifugation step, the viral pellet was now visible at the bottom of the tube. The supernatant was carefully aspirated and the viral pellet resuspended in 200 to 300 μ l of Hanks balanced salt solution (or phosphate-buffered saline) and frozen at -80°C until further use.

LPS stimulation of splenocytes. Splenocytes from transgenic and control animals were collected following mechanical dissociation of spleens. The resultant cell suspensions were freed of debris by passage through 40- μ m cell filters. Red blood cells were lysed by brief incubation with red blood cell lysis buffer (Sigma). Cells were subsequently plated on 150-mm plates and incubated at 37°C for 2 hours to allow adherence of phagocytes to plastic. Cells in suspension were then collected, and T cells were depleted following incubation with anti-CD90.2-loaded Dynabeads (Invitrogen). The extent of T-cell depletion was confirmed by flow cytometry: postdepletion, more than 92% cells were B220 positive (B220⁺), routinely. Cells were then resuspended in B-cell medium (RPMI 1640-10% fetal calf serum-2 mM β -mercaptoethanol) and supplemented with a combined antibiotic-antimycotic solution to give a 1 $\times$ final concentration (Gibco). Lipopolysaccharide (LPS; final concentration, 20 μ g/ml; Sigma), interleukin-2 (IL-2; final concentration, 10 ng/ml; R&D), and IL-6 (final concentration, 10 ng/ml; R&D) were added, and the cultures were incubated at 37°C in a humidified tissue culture incubator.

Flow sorting and retroviral transduction. Splenocyte suspensions from boosted transgenic animals were T cell depleted using anti-CD90.2-loaded Dynabeads (Invitrogen). mRFP-positive (mRFP⁺) cells were flow sorted to purity with a high-speed MoFlo sorter using a 568-nm laser. Sorted cells were resuspended in B-cell medium (RPMI 1640-10% fetal calf serum-2 mM β -mercaptoethanol), supplemented with a combined antibiotic-antimycotic solution to give a 1 $\times$ final concentration (Gibco). Polybrene was added to give a final concentration of 6 μ g per ml. Concentrated viral supernatant was added to 10% of the final volume (i.e., 100 μ l in 1 ml total medium). Cells (2×10^5 to 5×10^5) were added in each well of a 12-well plate. The plates were centrifuged in a tabletop centrifuge at 2,500 rpm for 90 min at room temperature. Following spin infection, cells were transferred in a tissue culture incubator and incubated at 37°C for approximately 1 hour. To assay for reporter gene expression, transduced cells were cultured for 48 to 72 h in the presence of LPS (10 μ g/ml; Sigma), IL-2 (10 ng/ml), IL-6 (10 ng/ml), and soluble CD40L (all cytokines were obtained from R&D). It should be noted that transductions of in vivo-activated, flow-sorted TVB-mRFP⁺ lymphocytes were carried out in the absence of LPS or added growth factors. Low-concentration LPS and growth factors were added after transduction to enhance the survival of transduced cells until the assay for reporter gene expression.

RESULTS

Generation of *Prdm1*:TVB-mRFP mice. Because the locations of the regulatory elements directing the tissue- and stage-specific expression pattern of *Prdm1* are unknown, we created BAC-based transgenes by homologous recombination in *E. coli*. The *recA*-mediated BAC modification method developed by Gong and colleagues (14) was employed to generate the *Prdm1*:TVB-mRFP transgene. In a two-step recombination process, the sequences encoding TVB, fused to the sequences encoding red fluorescent protein, were placed immediately downstream of the *Prdm1* translation initiation ATG codon (see Materials and Methods and Fig. S1 in the supplemental material for a detailed explanation of the modification process). Four independent lines of transgenic mice were derived following injection of the modified BAC transgene into fertilized oocytes. The BAC transgene was transmitted to progeny in a simple Mendelian pattern of inheritance and, in one transgenic line, was successfully bred to homozygosity. The BAC transgene was backcrossed into a pure C57BL/6J genetic background for 10 generations. *Prdm1*:TVB-mRFP-transgenic animals were indistinguishable from nontransgenic littermates in development, growth characteristics, fertility, and gross anatomical appearance upon dissection.

A small population of TVB-mRFP⁺ cells is detectable in hematopoietic tissue from mice mounting immune responses to T-cell-dependent antigens. To induce transcription of the *Prdm1*:TVB-mRFP transgene in immune cells, adolescent (6- to 8-week-old) transgenic mice and nontransgenic littermates were immunized with an intraperitoneal injection of sheep red blood cells. Immunization with sheep red blood cells provokes a T-cell-dependent immune response (17). Booster immunizations were administered beginning 2 to 3 weeks after primary immunization. Spleen and bone marrow samples were collected from immunized transgenic and control mice following booster immunization and analyzed by flow cytometry. *Prdm1*:TVB-mRFP hemizygous transgenic animals, but not nontransgenic littermates, exhibited a small population of cells in both spleen and bone marrow, emitting red fluorescence when excited by a 568-nm laser (Fig. 1). The size of this population ranged from less than 0.1% to approximately 0.5% of all cells in both spleen and bone marrow. The upper end of this range was observed in animals that had received two or more booster immunizations. The proportion of cells expressing the fusion receptor under these conditions was similar to the proportion of cells displaying green fluorescence when green fluorescent protein (GFP) sequences were inserted into the native *Prdm1* locus (22).

Immunophenotyping confirms lineage-restricted expression of TVB-mRFP in splenocytes. In order to characterize the mRFP⁺ population, we analyzed the surface immunophenotype (Fig. 1A). Splenocyte suspensions from transgenic and control animals, obtained 1 week following booster immunization with sheep red blood cells, were analyzed by staining with antibodies directed against surface markers characteristic of various hematopoietic lineages. CD138, a surface marker for mature plasma cells, was present on 20 to 60% of the mRFP⁺ population. This is in keeping with the key role of Blimp-1 in plasma cell ontogeny. A subpopulation of TVB-mRFP⁺ cells was also positive for B220, a pan-B-cell marker that is gradually downregulated upon plasmacytic differentiation. B220⁺/mRFP⁺ cells likely correspond to developing plasmablasts, transiting a differentiation stage intermediate between a late B cell and a plasma cell (2, 22), and may represent the precursor to *Prdm1*-expressing malignancies arising in the environment of the germinal center, such as plasma cell myeloma and some mature B-cell lymphomas (6).

Blimp-1⁺ T cells constitute a second immune effector subset rendered susceptible to gene transduction in our system. Approximately one-third to two-thirds of TVB-mRFP⁺ cells in immunized animals costained with an antibody against the pan-T-cell marker CD90.2 (not shown), and the vast majority of these were shown to express the T-helper-cell marker CD4 but not the T-cytotoxic-cell marker CD8 (Fig. 1A). *Prdm1*-expressing T cells include important subsets of antigen-experienced T cells, including effector and memory T cells and at least a subset of CD4⁺ CD25⁺ regulatory T cells (29).

TVB-mRFP⁺ cells did not significantly coexpress myeloid (Gr-1), erythroid (Ter119), and natural killer (DX-5) cell surface markers (Fig. 1A). These findings confirm the restricted pattern of expression of the TVB fusion receptor in our system, closely correlating with the known expression pattern of *Prdm1*.

Immunization results in expansion of TVB-mRFP⁺ T cells.

Splenocytes from age-matched (6- to 8-week-old) transgenic animals and nontransgenic controls that had not undergone prior immunization with sheep red blood cells were similarly analyzed by flow cytometry. The proportion of total mRFP⁺ splenocytes from nonimmunized animals was comparable to the low end of the range observed in spleens from immunized animals (in three nonimmunized, age-matched animals, mRFP⁺ splenocytes were 0.1%, 0.12%, and 0.13% of total splenocytes). However, important differences were seen in the lineage specification of mRFP⁺ cells, compared to what was found for immunized mice. In all three nonimmunized animals, the ratio of CD138⁺ mRFP⁺/CD90.2⁺ mRFP⁺ cells was approximately 2:1 (Fig. 1B). This ratio was typically inverted in immunized, age-matched transgenic animals in which most mRFP⁺ T cells coexpressed CD4 (Fig. 1A). These results demonstrate that immunization with sheep red blood cells results in expansion of a TVB-mRFP-expressing subpopulation of T cells. This subpopulation likely corresponds to *Prdm1*-expressing effector T cells generated in the context of a reaction mounted against sheep red blood cells, a T-cell-dependent immunogen.

In situ detection pattern of the TVB-mRFP fusion receptor in hematopoietic tissue.

We next sought to define the pattern of expression of the TVB-mRFP fusion receptor in relation to the architecture of hematopoietic organs and the location of cells producing endogenous Blimp-1. To do so, we used a polyclonal antibody against mRFP in frozen sections from hematopoietic tissues obtained from immunized transgenic and control animals, harvested at 1 week following booster immunization with sheep red blood cells. As shown in Fig. 2, TVB-mRFP⁺ cells were detected in small numbers in the spleens of immunized transgenic animals but not in nontransgenic controls. They were associated with splenic follicles, as well as a subset of periarteriolar lymphoid sheath areas, the latter constituting an area replete with T cells (Fig. 2A, B, and C). Scattered TVB-mRFP⁺ cells were seen within germinal centers, the sites of antibody affinity maturation, following exposure to antigen (Fig. 2A and B). In the extrafollicular areas of the spleen (collectively designated "red pulp" in Fig. 2), TVB-mRFP⁺ cells tended to group in small clusters (Fig. 2A, top). Other sites of detection of the fusion receptor correlated with known sites of clustering and migration of *Prdm1*-expressing immune effector cells. In mesenteric lymph nodes from immunized animals, TVB-mRFP⁺ cells were seen in association with lymph node cortical areas, including germinal centers, but also within draining areas of the lymph node, medullary cords, and sinuses (Fig. 2E). The last are known to contain large numbers of plasmacytic precursors. Only a few scattered mRFP⁺ cells were seen in the thymus (see Fig. S3 in the supplemental material). This is consistent with the low levels of reporter GFP fluorescence in developing thymocytes from mice with a GFP cDNA inserted into the native *Prdm1* locus (23). In the bone marrow, staining for TVB-mRFP was often seen in association with plasma cells, as assessed by morphological criteria (Fig. 2F). Lastly, we observed staining for mRFP in a population of cells located in the lamina propria of the small bowel, an area rich in immune effectors, including plasma cell precursors and regulatory T cells (see Fig. S3 in the supplemental material). The pattern of expression of the TVB-mRFP

FIG. 1. (A) Detection of the TVB-mRFP fusion receptor by FACS on hematopoietic cells from *Prdm1*:TVB-mRFP-transgenic, immunized animals. Splenocyte and bone marrow cell suspensions from an immunized animal hemizygous for the *Prdm1*:TVB-mRFP transgene, but not those from an immunized, nontransgenic littermate, display a small subpopulation in both spleen and bone marrow, emitting red fluorescence following excitation with a 568-nm laser. Live (DAPI-excluding) populations are gated in all graphs. Splenocytes from the transgenic animal were stained with a panel of antibodies directed against lineage- and stage-specific cell surface markers, and the proportion of mRFP⁺ cells coexpressing each marker was assayed. CD138 is expressed on mature plasma cells; B220 is expressed on B cells; CD4, CD8, and CD25 are expressed on subsets of T cells; Ter119 is expressed on erythroid cells; Gr-1 is expressed on myeloid cells; and DX-5 is expressed on NK cells. Baseline staining with an

FIG. 2. In situ detection of the TVB-mRFP fusion receptor. An antibody against red fluorescent protein was used to detect TVB-mRFP⁺ cells in hematopoietic tissue from *Prdm1*:TVB-mRFP-transgenic animals as well as nontransgenic littermate controls. (A, B, C) Spleen cryosections from an immunized *Prdm1*:TVB-mRFP transgenic animal. (D) Spleen cryosection from an immunized, nontransgenic littermate control. Immunization with sheep red blood cells provokes a T-cell-dependent immune response with formation of germinal centers (GC). Scattered mRFP⁺ cells (brown; 3,3'-diaminobenzidine tetrahydrochloride [DAB]) are seen within germinal centers as well as within the follicular mantle (FM). Multiple mRFP⁺ cells are also seen in the periarteriolar sheath (PALS), a region populated mostly by T cells. In contrast, no staining is seen on tissue from an immunized, nontransgenic control following incubation with the anti-mRFP antibody. Hematoxylin was used as a counterstain. RP, red pulp. Magnification, $\times 10$. (E) Mesenteric lymph node section from a *Prdm1*:TVB-mRFP transgenic animal. Multiple, scattered mRFP⁺ cells are seen in cortical areas (LnC) as well as draining areas of the lymph node, medullary cords (MC), and medullary sinuses (MS). Hematoxylin was used as a counterstain. Magnification, $\times 10$. (F) Bone marrow aspirate from a *Prdm1*:TVB-mRFP transgenic animal. The only cell staining for mRFP (brown; DAB) in this cluster has a plasmacytic form. Various other hematopoietic lineage cell types do not display detectable staining for mRFP. Hematoxylin was used as a counterstain. Magnification, $\times 100$. (G, H) Spleen cryosections were stained with an antibody against Blimp-1 (nuclear brown staining; DAB), followed by an antibody against mRFP (blue cytoplasmic/membrane staining; BCIP/NBT) from a transgenic animal (G) and a nontransgenic littermate control (H). Membrane staining for mRFP is seen in association with nuclear Blimp-1 staining on tissue obtained from the transgenic animal but not on that from the nontransgenic control. No counterstain was used. Magnification, $\times 100$. (I) Double staining for mRFP (brown; DAB) and Ki67 (blue; BCIP/NBT) on a lymph node cryosection from an immunized *Prdm1*:TVB-mRFP animal. Some, but not all, mRFP⁺ cells are in cell cycle, as denoted by costaining with an anti-Ki67 antibody (arrows).

fusion receptor in hematopoietic tissue is therefore consistent with that reported for *Prdm1*.

Expression of TVB-mRFP correlates with production of endogenous Blimp-1 protein. Given this restricted expression pattern, we sought to establish directly whether expression of TVB-mRFP fusion receptor correlated with production of en-

dogenous Blimp-1 protein. To this end, we costained cryosections of the spleens from immunized transgenic animals, as well as nontransgenic controls, with an antibody against mRFP, followed by staining with a monoclonal antibody against Blimp-1. The results are shown in Fig. 2G and H. There is a direct correlation between detectable, endogenous nuclear

isotype control (Ctrl) antibody as well as an unstained control is also presented. The proportion of mRFP⁺ splenocytes staining positive for each of the above-mentioned markers is shown in the upper right hand corner of each individual panel as a percentage of the total number of mRFP⁺ splenocytes. (B) Detection of the TVB-mRFP fusion receptor by FACS on splenocytes from *Prdm1*:TVB-mRFP-transgenic animals and nontransgenic controls that had not undergone prior immunization. Splenocyte suspensions from a nonimmunized, 8-week-old animal hemizygous for the *Prdm1*:TVB-mRFP transgene, but not those from a nontransgenic littermate, contain a small subpopulation emitting red fluorescence following excitation with a 568-nm laser. Live (DAPI-excluding) populations are gated in all graphs. Splenocytes from the transgenic animal were stained with an antibody against CD138 (a plasma cell marker) and an antibody against CD90.2 (a pan-T-cell marker). Baseline staining with an isotype control antibody is also presented. The proportion of mRFP⁺ splenocytes staining positive for each of the above-mentioned markers is shown in the upper right hand corner of each individual panel as a percentage of the total number of mRFP⁺ splenocytes.

Blimp-1 protein and staining for cytoplasmic/membrane mRFP in splenocytes from transgenic animals (Fig. 2G). Splenocytes from nontransgenic controls did not stain with the anti-mRFP antibody (Fig. 2H). Nuclear Blimp-1 staining was occasionally seen in the absence of membrane/cytoplasmic mRFP staining, likely reflecting a sensitivity limit for immunohistochemical detection of mRFP. Conversely, cytoplasmic/membrane staining for mRFP was only rarely seen in the absence of Blimp-1-nuclear staining. These results suggest that TVB-mRFP expression simulates production of endogenous Blimp-1 protein in this system.

A fraction of TVB-mRFP⁺ cells is in cell cycle. To determine the cycling status of TVB-mRFP⁺ cells, we performed double immunostaining with an antibody against mRFP and an antibody against the cell cycle marker, Ki67 (Fig. 2I). A small subset of mRFP⁺ cells was in cell cycle, as determined by costaining for Ki67. Ki67⁺ TVB-mRFP⁺ cells were seen in both the follicular and the extrafollicular areas of the spleen and both the cortical and the draining areas of the lymph node. Interestingly, they were more abundant in the draining areas of the lymph node, with a frequency as high as 10% of the total mRFP⁺ cells in some fields. Ki-67⁺ Blimp-1⁺ cells are thought to include young plasmablasts that retain a proliferative potential transiently prior to maturation and terminal differentiation into nondividing plasma cells (2).

Transduction of in vivo-activated, TVB-mRFP-expressing lymphocytes with ALV vectors bearing a subgroup B envelope.

We next defined conditions for ALV-mediated gene transfer into antigen-experienced, in vivo-activated B lymphocytes expressing the fusion receptor TVB-mRFP under *Prdm1* regulation. Splenocytes were harvested from BAC-transgenic, adult animals at 1 week following booster immunization with sheep red blood cells. The preparations were T cell depleted, and mRFP⁺ cells were flow sorted using a 568-nm-laser-equipped cell sorter. The sorted mRFP⁺ fractions were subsequently transduced with concentrated ALV retroviruses at a high multiplicity of infection (MOI) of 20 to 40. Following transduction, cells were cultured for 48 h to allow expression of the reporter gene. Cells gated in the lymphocyte light scatter gate were subsequently analyzed for reporter gene fluorescence by FACS (Fig. 3). Mouse lymphocytes required a very high MOI for efficient transduction and expression of exogenous genes. In contrast, human lymphoid cell lines RPMI8226 and ARH77 stably expressing TVB displayed fluorescent reporter gene activity following transduction at much lower MOIs (as low as 1 to 2; not shown).

To better understand the requirements for efficient virus-encoded reporter gene expression in primary lymphocytes, we directly compared two different ALV-based vectors. Both vectors are replication competent in avian, but not mammalian, cells. In the design of the first vector, GFP expression is driven by the native ALV long terminal repeat (LTR) through a message generated by splicing to an acceptor site just upstream of the inserted cDNA encoding GFP (Fig. 3A). In the second vector, GFP expression is driven by an internal cytomegalovirus (CMV) promoter. In this case, the splice acceptor site, located just upstream of the inserted cDNA, has been disrupted (Fig. 3B). Transduction of TVB-mRFP-expressing B lymphocytes with either vector at equivalent MOIs yielded comparable numbers of cells displaying reporter fluorescence,

likely reflecting an upper limit to infection efficiency dictated by the proportion of ALV receptor-expressing cells that were actively cycling under the conditions employed. However, the introduction of an internal CMV promoter appears to have generated a larger subpopulation of cells exhibiting extremely bright reporter fluorescence ($>10^4$ compared to the baseline). This may reflect an increased transcriptional activity of the CMV promoter, compared to that of the ALV LTR element, in murine lymphocytes. As a control for subgroup specificity, incubation of TVB⁺ cells with a vector expressing an ALV subgroup A envelope glycoprotein did not produce any cells displaying reporter gene fluorescence (Fig. 3C).

To challenge the assumption that expression of TVB is required for transduction of lymphocytes by ALV vectors bearing a subgroup B envelope glycoprotein, we have attempted to transduce in vitro-activated splenocytes from *Prdm1*:TVB-mRFP animals, in parallel to nontransgenic littermate controls, with an RCAS(*envB*)-GFP vector. As shown in Fig. S4 in the supplemental material, lymphocytes obtained from transgenic animals, but not lymphocytes obtained from nontransgenic controls, exhibited reporter GFP fluorescence following transduction. These results show that expression of TVB is required for transduction by ALV-type vectors bearing a subgroup B envelope.

DISCUSSION

We report here the generation of a novel in vivo system enabling the somatic and flexible genetic manipulation of Blimp-1-producing cells through ectopic expression of TVB, an ALV receptor, under the control of transcriptional elements belonging to the gene encoding Blimp-1, *Prdm1*. We created this system because perturbation of the growth and/or homeostasis of *Prdm1*-expressing hematopoietic cells has been directly linked with disease processes, such as tumors of the B-cell lineage and autoimmunity (29). Moreover, expression of *Prdm1* has been shown to play a central role in the lineage commitment of specific embryonic and adult lineages, some with stem cell properties. An animal system that enables the flexible genetic manipulation of *Prdm1*-expressing cells could be a valuable tool for investigating these diverse physiologic and pathological processes in vivo.

To achieve cell-specific transduction of lineages regulated by Blimp-1, we ectopically expressed the ALV receptor TVB, driven by regulatory elements belonging to the *Prdm1* locus. This is the first transgenic system employing the ALV receptor TVB; among the various ALV receptors, only the receptor TVA has previously been utilized for tissue-specific gene delivery in mammalian systems (13, 33). TVB does not share structural similarity with TVA and is encoded by a separate locus in the chick genome. The expansion of the spectrum of ALV receptors that can be used for generation of mammalian transgenic systems is important because it will allow lineage-directed gene targeting in the same animal following gene transduction through alternate ALV receptors. We envisage several attractive applications of this technology: it can be used to address cell-of-origin questions in cancer; it can also provide an attractive approach to probing the complex interactions between tumor cells and nonmalignant cells in the microenvironment.

FIG. 3. Transduction of TVB-mRFP-expressing lymphocytes with ALV-based retroviral vectors. High-titer retroviral stocks of two different ALV vectors, both bearing a subgroup B envelope (encoded by *envB*), were used to transduce a reporter gene cDNA (*GFP*) into flow-sorted TVB-mRFP⁺ splenocytes and compared to an ALV vector bearing a subgroup A envelope. In all cases, the viral titers were 10⁸ infectious particles/ml of viral stock, as assayed by limited dilution on chick embryonic fibroblasts (DF-1 cells). Transduced cells were assayed for green fluorescence at 48 h posttransduction. Cells in the lymphocyte light scatter gate were used for analysis. (A) An RCAS-type ALV vector, bearing a subgroup B envelope, drives expression of the inserted cDNA (*GFP*) from the 5' viral LTR. In mammalian cells, the predominant message originates in the LTR and is spliced to a splice acceptor site just upstream of the insert (solid line). Two other mRNA species are efficiently produced in avian cells but not in mammalian cells (broken lines) (13): a full-length message (serving as genomic RNA for retroviral progeny) and a spliced, envelope-encoding message. (B) An RCAN-type vector, bearing a subgroup B envelope, drives expression of *GFP* from an internal CMV promoter following disruption of the splice acceptor site immediately upstream of the insert (solid line). In avian cells, full-length and envelope-encoding messages are also produced (broken lines). (C) An RCAN-type vector similar to that for panel B, but bearing a subgroup A envelope gene, was used as a control. Closed diamond, splice donor site; open diamond, splice acceptor site.

To trace the expression of TVB in this system, we expressed the ALV receptor as a fusion to mRFP. The mRFP moiety of the fusion receptor was readily detectable by flow cytometry with a 568-nm laser. Using this approach, we detected TVB-

mRFP expression in a small subpopulation of splenocytes and bone marrow cells in young animals undergoing immune responses to sheep red blood cells, a T-cell-dependent immunogen. Analysis of the TVB-mRFP-expressing population by sur-

face immunophenotype in immunized animals confirmed that the majority consisted of CD138⁺ plasma cells and CD4⁺ T cells. Strikingly, we did not find a significant proportion of CD8⁺ T cells coexpressing TVB-mRFP. This may be due to the specific conditions employed to induce T-cell-dependent immune responses, i.e., the immunization of young animals with sheep red blood cells. We postulate that different conditions, such as viral challenge, may elicit a substantially higher proportion of CD8⁺ mRFP⁺ effector cells (5, 10). The fluctuating makeup of the mRFP⁺ population under diverse experimental conditions is further underscored by the finding that in nonimmunized, age-matched animals, the T-cell component of this population was relatively underrepresented (Fig. 1B). We also detected a reproducible B220⁺ population coexpressing TVB-mRFP. We postulate that at least a component of this population may include proliferative plasma cell precursors generated in the course of T-cell-dependent immune responses. These cells, in turn, may constitute the precursor of *Prdm1*-expressing B-cell malignancies arising in the germinal center, such as plasma cell myeloma and some diffuse large B-cell lymphomas.

We subsequently defined conditions for efficient transduction of *Prdm1*-expressing lymphocytes by ALV vectors incorporating a subgroup B envelope. To our knowledge, this is the first ALV-based system, and one of the few retroviral systems, allowing efficient retroviral transduction of in vivo-activated mature lymphocytes (21, 24). Because only a minority of TVB-expressing cells are in cell cycle (e.g., most CD138⁺ cells are quiescent plasma cells), the fraction of susceptible target cells transduced under the conditions delineated in this paper is likely to be significantly higher than the proportion (5%) of total cells transduced (Fig. 3). We determined that a high MOI (20 to 40) is required for detection of reporter fluorescence in transduced primary lymphocytes but that transduction at a much lower MOI was adequate to detect reporter fluorescence in established human lymphoid cell lines engineered to stably express TVB. Furthermore, we found that the incorporation of an internal CMV promoter in the viral construct appears to allow a subset of cells to exhibit extremely high levels of reporter fluorescence; similar levels of reporter GFP fluorescence were not obtained when expression of the reporter gene was driven by the native ALV LTR. This finding may be relevant to experiments in which high levels of gene expression are desired.

We are initially using *Prdm1*:TVB-mRFP transgenic animals as a basis for developing a flexible animal model for multiple myeloma, an incurable cancer of plasma cells that has hitherto proven difficult to model faithfully. More generally, in the B-cell lineage, genetic manipulation of *Prdm1*-expressing effectors could further illuminate the molecular processes underlying commitment to a plasmacytic fate (and likely the exclusion of a memory B-cell fate). The role of Blimp-1 in T-cell maturation has only recently begun to be characterized; however, expression of this transcription factor has already been linked with differentiation, homeostasis, and/or function of memory T cells and at least a subset of regulatory T cells. The spectrum of lineages rendered susceptible to genetic manipulation in the system presented here is likely to extend to other adult and embryonic lineages regulated by Blimp-1. Thus, *Prdm1*:TVB-mRFP mice could prove a useful tool for

studying several aspects of hematopoiesis, immunity, development, and cancer that are associated with production of Blimp-1.

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F.A. and H.E.V. designed the study, analyzed the data, and wrote the manuscript. F.A. performed the experiments.

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