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Targeting Rare Populations of Murine Antigen-Specific T Lymphocytes by Retroviral Transduction for Potential Application in Gene Therapy for Autoimmune Disease[1]

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CD4$^+$ T cells are important mediators in the pathogenesis of autoimmunity and would therefore provide ideal candidates for lymphocyte-based gene therapy. However, the number of Ag-specific T cells in any single lesion of autoimmunity may be quite low. Successful gene transfer into autoantigen-specific CD4$^+$ T cells would serve as an ideal vehicle for site-targeted gene therapy if it were possible to transduce preferentially the small number of autoantigen-specific T cells. In this study we have demonstrated that retroviral infection of CD4$^+$ lymphocytes from either autoantigen-stimulated TCR transgenic mice, or Ag-activated immunized nontransgenic mice, with a retroviral vector (pGCIRES), resulted in the transduction of only the limited number of Ag-reactive CD4$^+$ T cells. In contrast, polyclonal activation of the same cultures resulted in transduction of non-antigen-specific lymphocytes. Transduction of Ag-reactive CD4$^+$ T cells with pGCIRES retrovirus encoding the regulatory genes IL-4 (IL4) and soluble TNF receptor (STNFR) resulted in stable integration and long-term expression of recombinant gene products. Moreover, expression of the pGCIRES marker protein, GFP, directly correlated with the expression of the upstream regulatory gene. Retroviral transduction of CD4$^+$ T cells targeted specifically Ag-reactive cells and was cell cycle-dependent and evident only during the mitosis phase. These studies suggest that retroviral transduction of autoantigen-specific murine CD4$^+$ T cells, using the pGCIRES retroviral vector, may provide a potential method to target and isolate the low frequency of autoantigen-specific murine CD4$^+$ T cells, and provides a rational approach to gene therapy in animal models of autoimmunity. ***The Journal of Immunology*****, 2000, 164: 3581–3590.**

Autoantigen-specific CD4$^+$ T lymphocytes have been implicated in the pathogenesis of autoimmune diseases (1–6). Tissue-specific homing properties of autoantigen-specific CD4$^+$ T cells suggested that these cells might be ideal vehicles for delivery of retroviral-encoded regulatory proteins in a site-specific manner as a therapy for autoimmune diseases. In many autoimmune diseases, disease pathogenesis is mediated by Ag-specific CD4$^+$ T cells that secrete Th1 pro-inflammatory cytokines (7, 8). Systemic administration of Th2 "regulatory" cytokines, which serve to counter the effects of the pro-inflammatory Th1 cytokines, has previously been shown to ameliorate autoimmune diseases (9–11). However, the inherent problems of nonspecific toxicity limit the usefulness of systemic cytokine delivery as a potential therapy. Therefore, a rational approach to treating autoimmune diseases might include manipulation of autoantigen-specific T cells for the delivery of regulatory (Th2-type) cytokines to autoimmune lesions (12–17).

Varied approaches to alter or abrogate inflammatory T cell responses in autoimmune disease states have been explored. Among the most promising are those that exploit T cells for delivery of therapeutic proteins (18–21). Early studies from our laboratory, using an animal model of the human disease multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), demonstrated that autoantigen-specific T cell hybridomas, transduced using retroviral vectors containing genes encoded for regulatory cytokines were capable of delivering cytokines to the autoimmune lesions in the CNS (22, 23). Disease could be ameliorated or exacerbated depending on the cytokine that was delivered. Transduced T cell hybridomas, which lacked the tissue-specific TCR for Ag but secreted the same anti-inflammatory cytokines, were ineffective. Thus, tissue-specific homing and/or retention was required for therapeutic effect. More recent studies have used transduced autoantigen-specific T cell clones to deliver immunosuppressive proteins to autoimmune lesions of EAE (24). These studies demonstrated that expression of retroviral-encoded regulatory cytokines did not alter the homing abilities of transduced T cell hybridomas or T cell lines, and, importantly, demonstrated that T cells, transduced by retrovirus for the expression of regulatory genes, had the potential to deliver these proteins in a site-specific manner. If nontransformed, primary T cells could be used to replace T cell hybridomas and T cell lines, then transduction of autoantigen-specific T cells would represent a viable therapeutic strategy for the treatment of autoimmune disease.

Primary murine CD4$^+$ T cells have been extremely difficult to infect with retroviruses, and are more often refractory to retroviral transduction. However, data presented in this paper demonstrate

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efficient and robust retroviral transduction of Ag-specific murine $CD4^+$ T cells from naive TCR transgenic or conventional Ag-challenged mice using the pGCIRES vector. Studies using "conventional" mice primed with foreign peptide confirmed that the small population of Ag-specific $CD4^+$ T cells could be targeted by retroviruses for transduction and then isolated using the pGCIRES marker protein, GFP (green fluorescent protein).[3] The development of stable retroviral-mediated transduction of autoantigen-specific murine $CD4^+$ T cells should provide a novel method to alter T cell function and is of particular interest for the application of gene therapy in autoimmune disease.

Materials and Methods

Mice

PL/J and DBA/2 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B10.PL mice, transgenic for a TCR reactive with myelin basic protein (MBP) NAc_{1-11}, have been described elsewhere (25) and were obtained from Dr. Caroline C. Whitacre (Ohio State University, Columbus, OH). Mice were used between 6 and 12 wk of age and maintained in the Department of Comparative Medicine, Stanford Medical Center.

Plasmids

The pGCIRES retroviral plasmid was constructed using the pLCAT backbone with the 5′ and 3′ long terminal repeats (LTR) and the psi (ψ) packaging sequence from the Moloney murine leukemia virus (MMLV)-based MFG vector (provided by Dr. R. C. Mulligan). A 722-bp enhanced codon-substituted humanized form of green fluorescent protein (huGFP) with site-specific mutations (S66T, V164A, H232L, and a G to A base change at residue 69) to the native jellyfish (*Aequoria victoria*) protein was used as the reporter protein (provided by Dr. M. A. Anderson, Stanford University) and was inserted into the retroviral plasmid by *Bam*HI and *Nco*I digestion (New England Biolabs, Beverly, MA). The encephalomyocarditis virus internal ribosome entry site (EMCV IRES) from pWZLBleo (provided by P. J. Morgenstern) was cloned upstream of the GFP by *Sna*BI/*Nco*I to *Pml*I/*Nco*I (insert to vector) restriction ligation. The multiple cloning site from the pCR-Script Amp plasmid (Stratagene, La Jolla CA) was amplification with standard T3 and T7 oligonucleotide primers and cloned upstream of the EMCV IRES into the T4 DNA polymerase-treated *Sal*I site. The final ampicillin-resistant bicistronic retroviral reporter construct was 6691 bp and termed pGCIRES. The pWTGFP plasmid is a murine stem cell virus-based retroviral vector and contained the puromycin resistance gene, polio IRES, and wild-type GFP. The plasmid Ca116 mp60hg4 contained the murine p60 TNF receptor (TNFR) subunit linked to the human IgG4 Fc domain provided by Dr. Rachael Ettinger (Stanford University, Stanford CA) and Dr. J. Browning (Biogen, Cambridge MA). The soluble TNFR (sTNFR) fragment (1381 bp) was subcloned into pcDNA3 (Invitrogen, Carlsbad, CA) and was obtained by *Eco*RV/*Xho*I digestion and directionally cloned into pGCIRES between the *Srf*I and *Xho*I sites and termed pGCIRES.sTNFR (8072 bp). The murine IL-4 gene (447 bp) was amplified by PCR and subcloned into pGCIRES using *Srf*I restriction ligation and termed pGCIRES.IL4 (7138 bp).

Cell lines

The Phoenix retroviral producer cell lines are derived from a 293T cell line and have been described elsewhere (26). Ecotropic packaging cells (Phoenix-E) were cultured in DMEM complete (DMEM-C) that contained DMEM (Life Technologies, Gaithersburg MD) supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Retroviral producer lines were cultured in DMEM-C medium that contained diphtheria toxin (1 μg/ml) (Calbiochem, La Jolla, CA) for selection of the ecotropic envelope gene and hygromycin B (300 μg/ml) (Boehringer Mannheim, IN) for selection of the gag-pol genes. The NIH 3T3 cell line (ATCC CCL92; American Type Culture Collection, Manassas, VA) was cultured in DMEM supplemented with 10% bovine serum (JRH Biosciences, Lenexa KS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cells were maintained at 37°C in 6% CO_2.

Transfection assay

A total of 2.0×10^6 Phoenix-E packaging cells were cultured in 12 ml DMEM-C in 100-mm tissue culture dishes (Nalge Nunc International, Roskilde, Denmark). Following overnight incubation, the packaging cells were transfected with 10 μg retroviral plasmid DNA (Qiagen, Valencia, CA) or no plasmid DNA (mock) using a modified version of the calcium phosphate precipitation protocol described elsewhere (26). At 8–12 h post-transfection, calcium phosphate-containing medium was replaced with DMEM-C medium and cultures were maintained at 37°C in 6% CO_2 for 24–48 h, then at 32°C in 6% CO_2 for 16–24 h. Viral supernatant from transient transfections was harvested and filtered using a 0.45 μm filter (Nalge, Rochester NY), then stored at −80°C. Virus titers were determined for the Phoenix-E retroviral packaging cell lines using NIH 3T3 lines, as previously described (27). Virus stocks with titers $>4 \times 10^6$ were used for transduction of murine cells.

Ag-specific $CD4^+$ populations

Peptides of sperm whale myoglobin (SWM, 110–121) (AIIHVLHSRHPG) and MBP (NAc_{1-11}) (NAc-ASQKRPSQRHG) were synthesized and HPLC purified at the Protein and Nucleic Acid Facility (Beckman Center, Stanford University). Immunization of DBA/2 mice for production of Ag-reactive $CD4^{high}$ cells was conducted as previously described (28). Briefly, groups of 5–10 mice were immunized s.c. at the base of the tail with 100 μg SWM peptide in 100 μl IFA plus 10 μg/ml heat-killed *Mycobacterium tuberculosis* H37RA (Difco Laboratories, Detroit MI). At 8–10 days (or as indicated) postimmunization, single cell suspensions ($2.0–5.0 \times 10^6$ cells/ml) from either spleen or draining inguinal lymph nodes were cultured for transduction or stimulated in vitro before transduction for time periods indicated with Ag (10 μM SWM). Single cell splenocyte preparations (5.0×10^6 cells/ml) from MBP TCR transgenic mice were stimulated in vitro for 24 h with Ag (MBP NAc_{1-11}, 6 μM) or PMA (50 ng/ml) and ionomycin (1 μM). T cell medium (RPMI complete, RPMI-C) consisted of RPMI 1640 (Life Technologies) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% nonessential amino acids, 1 mM sodium pyruvate, 10 mM HEPES buffer, 50 μM 2-ME, and 10% FCS (HyClone, Logan UT). Cells were maintained at 37°C with 6% CO_2.

Infection assay

A total of $2.0–5.0 \times 10^6$ cells in 0.5 ml RPMI-C were plated in duplicate in 6-well plates (Falcon Labware, Franklin Lakes, NJ) and overlaid with 2 ml thawed recombinant retroviral supernatant supplemented with protamine sulfate (8 μg/ml) (Sigma, St. Louis MO). Plates were centrifuged at 2500 rpm at 32°C for 2 h and transferred to incubation at 32°C in 6% CO_2 for 16 h, or at time periods indicated. Medium was exchanged with RPMI-C medium supplemented with 10 U/ml murine recombinant IL-2 (R&D Systems, Minneapolis, MN) and then transferred to 37°C in 6% CO_2 for an additional 24 h. At 48 h postinfection, cells were analyzed for transduction by flow cytometric analysis of GFP expression.

FACS

Murine cells transduced with recombinant retrovirus cells were stained with rat anti-mouse CD4-PE (Caltag Laboratories, San Francisco CA). Dead cells were excluded using propidium iodide (PI) staining (Sigma) and live cells were analyzed for CD4-PE (PE channel) and GFP reporter expression (FITC channel). Analysis was performed on a FACScan cytometer (Becton Dickinson, Mountain View, CA). For $CD4^{high}$ T cell analyses, cells were stained with CD4-PE and sterile sorted into $CD4^{normal}$ or $CD4^{high}$ populations using a FACStar flow cytometer (Stanford FACS Facility, Stanford University). Sorted sample populations were reanalyzed following the initial collection to confirm sample fraction purity and CD4 expression (>95%). All data were analyzed using FlowJo (version 2.7.8) flow cytometry software (Tree Star, San Carlos CA).

Cell cycle analysis

For nonvital cell cycle analyses, cell samples ($1–3 \times 10^6$) were pelleted and resuspended in 200 μl buffer containing PBS and 2% FCS (FACS buffer). Single cell suspensions were resuspended in chilled 70% ethanol (2 ml) and incubated at least 18 h at 4°C for fixation. Cells were then pelleted and resuspended by passing through a 25-gauge needle into 800 μl of FACS buffer containing 100 μg/ml RNase and 40 μg/ml PI. Cell cycle distributions were analyzed by flow cytometry using linear scales with PI staining as a measure of DNA content. For vital cell cycle analyses, the cell samples were pelleted and resuspended in FACS buffer (1 ml) and stained with rat anti-mouse CD4-PE at a 1:100 dilution (Caltag Laboratories) for 30 min on ice. Hoechst 33342 (10 μg/ml) (Molecular Probes, Eugene OR) and verapamil (50 μg/ml) (Sigma) was added before incubation at 37°C for

[3] Abbreviations used in this paper: GFP, green fluorescent protein; MMLV, Moloney murine leukemia virus; LTR, long terminal repeat; ψ, psi packaging signal; EMCV, encephalomyocarditis virus; IRES, internal ribosome entry site; MBP, myelin basic protein; SWM, sperm whale myoglobin; sTNFR, soluble TNF receptor; PI, propidium iodide.

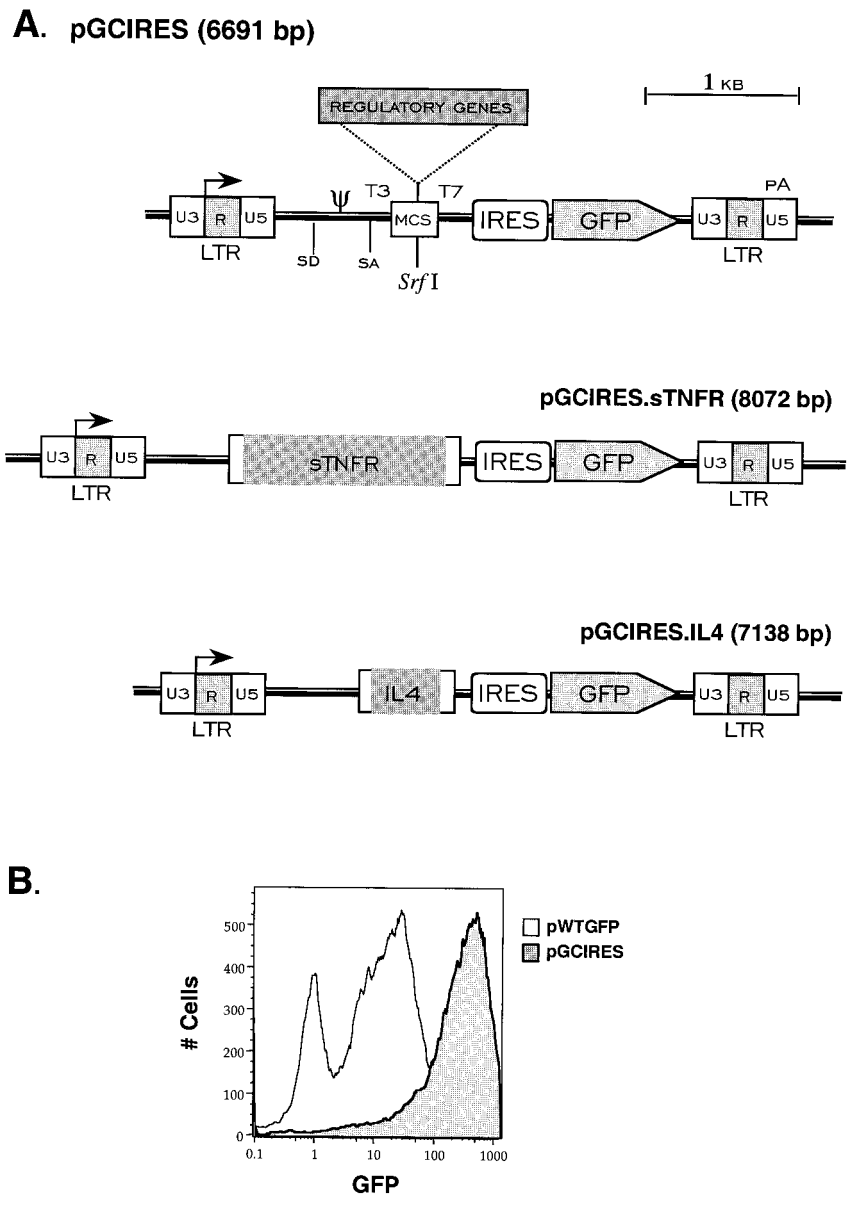

FIGURE 1. Schematic diagram of MMLV-based retroviral vectors. *A*, pGCIRES (6691 bp), retroviral vector containing the MMLV-MFG retroviral packaging signal (ψ) and 5′ and 3′ LTRs, a *Srf*I-containing multiple cloning site, the EMCV IRES, and a mammalian codon-enriched GFP variant. Arrows indicate transcriptional start site and direction of transcription. The pGCIRES.sTNFR (8072-bp) retroviral vector contains the murine p60 sTNFR. The pGCIRES.IL-4 (7138-bp) retroviral vector contains the murine IL-4 gene. *B*, Enhanced GFP reporter expression in murine NIH 3T3 cells transduced with recombinant retrovirus expressing either wild-type GFP (pWTGFP) or the mammalian codon enriched variant of GFP (pGCIRES). GFP expression was measured using flow cytometry with excitation at 488 nm and emission at 534 nm read as fluorescein. Histograms represent cell number as a function of GFP fluorescence. Positive peaks represent 62% and 95% infection of the total population for pWTGFP and pGCIRES, respectively.

1 h. Following incubation, samples were transferred to ice and kept in the dark before analysis using flow cytometry. Cells were analyzed and sorted using linear scales, and DNA content was measured by monochlorobimane and Hoechst 33342 lasers. The cells were fractionated into three populations: G_0/G_1, $S+G_2/M$, or M. The median third (33%) of the G_0/G_1 and $S+G_2/M$ populations and distal third (66–99%) of the M population was collected (5×10^5 cells collected per fraction). Sorted sample populations were reanalyzed following the initial collection to confirm sample fraction purity and CD4 expression (>95%).

ELISA analysis

IL-4 production was measured by a standard sandwich ELISA protocol. Supernatants were harvested 48 h after in vitro stimulation with 5 μg/ml MBP NAc_{1-11}. Capture anti-IL-4 Ab (11B11, 1 μg/ml) (PharMingen, San Diego, CA) was incubated in 96-well Maxisorp ELISA plates (Nalge Nunc) at 4°C overnight. After washing and a 30-min incubation with 5% FCS in PBS at room temperature, 50 μl of sample or standard dilution of murine rIL-4 (PharMingen) was added. Plates were incubated overnight at 4°C, and biotinylated anti-IL-4 (1BVD6-24G2, 0.5 μg/ml) (PharMingen) was added for 3 h at room temperature following washing. Plates were washed and streptavidin/peroxidase (Sigma) was added for 30 min. Wells were developed with 3,3′,5,5′-tetramethyl-benzidine substrate (Sigma), and the reaction was stopped with 1 N HCl. Plates were read at 450 nm on a microtiter plate reader (Wallac, Gaithersburg, MD). Cytokine concentrations (pg/ml) were determined by comparing the OD of samples to the standard curve.

Results

pGCIRES retroviral expression vectors

Transduction is a process following infection by retroviral vectors that allows chromosomal integration of the transferred genes and provides stable cellular gene expression. In experiments described below, we used the retroviral vector, pGCIRES (an MMLV-based vector) engineered to contain a multiple cloning site for incorporation of genes expressing "regulatory" proteins and a downstream selectable marker, GFP (Fig. 1*A*). The IRES sequence contains a portion of the 5′ untranslated region from the EMCV to allow cap-independent translation (29, 30). Incorporation of the IRES sequence allowed the formation of bicistronic RNA transcripts from a single promoter for expression of both the upstream gene, as well as the downstream reporter gene. The strong promoter and

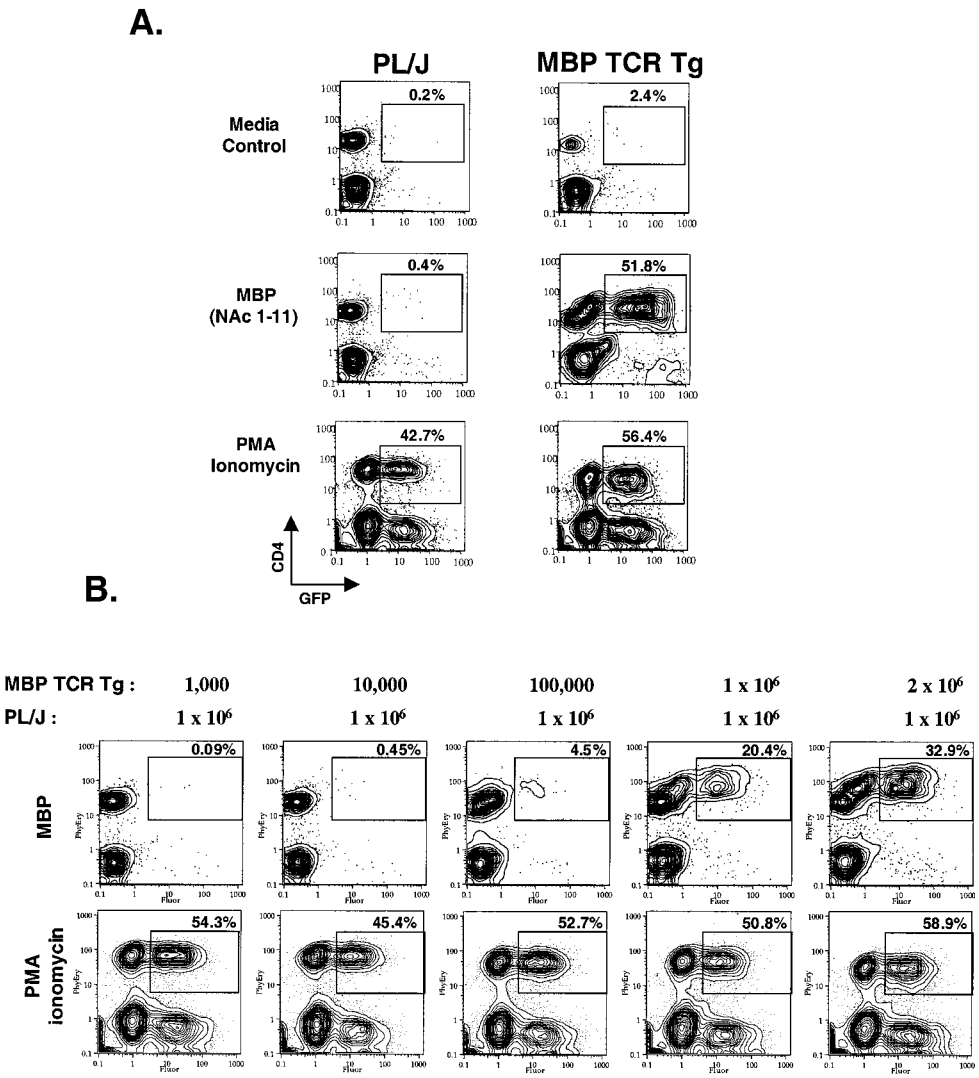

FIGURE 2. Preferential transduction of autoantigen-reactive $CD4^+$ T cells. *A,* Splenocytes from MBP TCR transgenic mice (MBP) or nontransgenic PL/J mice (PL/J) were cultured in vitro with 5 μg/ml (MBP NAc_{1-11}), 50 ng/ml PMA and 1 μM ionomycin (PMA/Ionomycin), or with medium alone (Media control). At 24 h poststimulation, splenocytes were infected with pGCIRES recombinant retrovirus. At 48 h postinfection, transduced splenocyte populations were stained using CD4-PE and analyzed by flow cytometery for expression of GFP. Gated regions (boxed outlines) represent transduction efficiencies (% infected) of $CD4^+$ T cells from the total $CD4^+$ cell population. *B,* Cell transduction with pGCIRES recombinant retrovirus is directly proportional to the frequency of Ag-specific $CD4^+$ T cells in mixed cultures. A total of 1×10^6 cells PL/J splenocytes (PL/J) were cultured with increasing numbers (1000 to 2×10^6 cells) of syngeneic MBP TCR transgenic cells (MBP) and stimulated with Ag (MBP NAc_{1-11}) or polyclonal activation (PMA/Ionomycin). Gated regions (boxed outlines) represent transduction efficiencies (% infected) of $CD4^+$ T cells from the total $CD4^+$ cell population.

enhancer domains in the 5′ LTR of this vector resulted in constitutive production of the bicistronic viral RNA transcript. As regulatory proteins, the murine Th2 cytokine IL-4 or the Ig fusion protein for the murine sTNFR were subcloned into the pGCIRES vector (pGCIRES.sTNFR and pGCIRES.IL4) (Fig. 1*A*). The pGCIRES-based retroviral vectors were used for subsequent transduction experiments.

Enhanced GFP reporter expression

Previously, reduced GFP emission levels associated with low integration frequency in murine retroviral-mediated gene transfer experiments has made GFP selection of transduced murine cells extremely difficult (31). In these studies, we have utilized an enhanced GFP marker gene with site-specific mutations in the native jellyfish gene (using human codon preferences) that augmented fluorescence when expressed in mammalian cells. Recombinant retrovirus prepared from retroviral vectors that contained the wild-type GFP (pWTGFP) or the humanized GFP variant (pGCIRES) were used to infect murine 3T3 cells. Infected cells were analyzed by flow cytometry for GFP expression as a measure of cell transduction. The mean channel of fluorescence for GFP emission in murine cells using the pGCIRES recombinant retrovirus was approximately three orders of magnitude higher than background levels, and two orders of magnitude higher than the fluorescence emission using wild-type GFP (pWTGFP) recombinant retrovirus (Fig. 1*B*).

Retroviral transduction of TCR transgenic autoantigen-specific $CD4^+$ T lymphocytes

Because it has been well established that retroviruses productively infect only dividing cells, we asked whether retroviral infection could transduce Ag-activated $CD4^+$ T cells transgenic for a TCR that recognized a known Ag. As a model system, we used B10.PL mice transgenic for a TCR reactive with MBP (NAc_{1-11}). $CD4^+$ T cells from MBP-TCR transgenic mice are >95% Ag specific ($V\beta8.2^+$) (25). To assess retroviral transduction in naive Ag-reactive $CD4^+$ T cells, splenocytes from either nontransgenic PL/J or MBP-TCR transgenic mice were stimulated in vitro with Ag (MBP peptide NAc_{1-11}) or with PMA and ionomycin, then exposed to pGCIRES recombinant retrovirus (Fig. 2*A*). Polyclonal activation using PMA and ionomycin stimulation resulted in nonspecific transduction of both CD4 positive and CD4 negative lymphocytes, and included $CD8^+$ T cells, $B220^+$ B cells and $GR1^+$ granulocytes (data not shown). However, stimulation with MBP limited transduction to the $CD4^+$ T lymphocytes (51.8%). Subsequent analysis of Ag-stimulated cultures using a transgene-specific TCR marker (anti-Vβ8.2), demonstrated that the transduced, GFP

Table I. *Transduction efficiencies for Ag-activated PL/J and MBP TCR transgenic splenocyte populations*

No. of Splenocytes[a]		Percentage of MBP Cells	Experimental Transduction Efficiency	Observed Transduction Efficiency
PL/J	MBP			
0	1×10^6	100		~50
1×10^6	0	0		<0.5
1×10^6	1000	0.1	0.05	0.09
1×10^6	10,000	1.0	0.5	0.45
1×10^6	100,000	10.0	5.0	4.5
1×10^6	1×10^6	50.0	25.0	20.4
1×10^6	2×10^6	66.6	33.3	32.9

[a] Retroviral transduction was performed on naive splenocyte cultures from PL/J and/or MBP TCR transgenic mice. Expected transduction efficiencies were based on the observation of 50% transduction efficiency of Ag-activated MBP-specific splenocytes infected with retrovirus in the absence of PL/J splenocytes. Efficiencies of cells transduced are reported as percentages of $CD4^+/GFP^+$ double positive T cells.

expressing cell population was limited to the TCR Vβ8.2 MBP-specific $CD4^+$ T lymphocytes (48.8%) and not due to the transduction of non-T cell $CD4^+$ populations ($CD11b^+$, <2%; and $NK1.1^+$, <1%) (data not shown). In contrast, control populations of nontransgenic PL/J splenocytes stimulated in vitro with the MBP peptide were not demonstrably transduced (0.4%).

To further demonstrate that retroviral transduction was specific for the Ag-activated TCR transgenic $CD4^+$ T cells, and to ask whether it was possible to transduce Ag-specific $CD4^+$ T cells at a frequency representing Ag-reactive cells in an immune lymph node (1/10,000), titrating amounts of splenocytes from MBP TCR transgenic mice ($H\text{-}2^u$) were cocultured with a fixed number of splenocytes from PL/J mice ($H\text{-}2^u$). Data presented in Fig. 2*B* represent cultures set up in parallel with a constant number of PL/J splenocytes and increasing numbers of MBP TCR transgenic splenocytes. Cultures were then stimulated in vitro with specific Ag (MBP) or with polyclonal activation (PMA and ionomycin) for 24 h and exposed to pGCIRES retrovirus. Because the transduction efficiency in this representative experiment of MBP-specific $CD4^+$ T cells was ~50% (Fig. 2*A*), the expected frequency of transduced $CD4^+$ T cells represented approximately half the number of MBP-specific transgenic splenocyte cells seeded into the mixed population (Table I). As anticipated for the Ag-activated populations, the frequency of transduced $CD4^+$ T cells would represent approximately half of the Ag responsive transgenic TCR-bearing MBP-specific splenocytes present in the total population (Fig. 2*B*). Analyses using the transgene-specific TCR marker (Vβ8.2) confirmed that the GFP-expressing cell populations were limited to the Vβ8.2 MBP-reactive $CD4^+$ T lymphocytes (data not shown). Conversely, populations stimulated by polyclonal activation showed no selective transduction of Ag-specific (Vβ8.2) $CD4^+$ T cells. Moreover, polyclonal activation resulted only in the transient transduction of murine splenocytes due to activation-induced cell death exhibited by PMA and ionomycin-stimulated populations shortly following the transduction analysis (data not shown). Importantly, these data demonstrated that stimulation of murine T cells with specific Ag resulted in stable retroviral transduction of only the Ag-reactive T cells. Subsequent experiments in our laboratory confirmed that $CD4^+$ T cells, stimulated with specific Ag and targeted for transduction using pGCIRES retrovirus, could be efficiently expanded in vitro by restimulation at 7- to 10-day intervals with peptide and irradiated APC (data not shown). In experiments conducted with four rounds of restimulation, aliquots of transduced MBP-reactive $CD4^+$ T cell lines were analyzed by flow cytometry for GFP reporter expression and assayed by ELISA for expression of cloned regulatory products and confirmed that retroviral-introduced transgene expression was stable for the duration of the experiments (>40 days) with peak transgene expression occurring 48–72 h following each round of restimulation. Moreover, the transduction of retroviral elements and the expression of recombinant transgenes did not alter the cytokine profile or the cell surface phenotype of the activated $CD4^+$ T cell (G. L. Costa and J. M. Benson, unpublished results).

Upstream gene expression correlates with downstream GFP reporter expression

Due to the therapeutic potential of tissue-specific expression of regulatory proteins in animal models of autoimmunity, pGCIRES retrovirus expressing the regulatory cytokine, IL-4, was used to infect splenocytes from MBP TCR transgenic mice. Confirmation of augmented fluorescence using the enhanced GFP variant in murine $CD4^+$ T cells was evidenced by a 20-fold higher GFP emission fluorescence, over background levels, in pGCIRES-transduced MBP TCR transgenic $CD4^+$ T cells. In contrast, the wild-type GFP recombinant retrovirus exhibited only a 2-fold increase in fluorescence emission over background levels (data not shown). Several independent experiments evaluating the infection of splenocytes from MBP TCR transgenic mice with either pGCIRES or pGCIRES.IL-4 retrovirus demonstrated transduction efficiencies as high as 80% (data not shown). Studies to evaluate the ability of the IRES element to allow GFP reporter expression to be used as an indicator of upstream gene expression were conducted using the pGCIRES.IL-4 recombinant retrovirus. Splenocytes from MBP TCR transgenic mice were stimulated in vitro with MBP peptide, and infected with pGCIRES, pGCIRES.IL-4, or mock recombinant retrovirus. Consistent with previous analyses, only the Ag-specific $CD4^+$ T cells from Ag-activated splenocyte cultures were transduced (Fig. 3*A*). Transduction efficiencies of the $CD4^+$ T cell populations infected with pGCIRES and pGCIRES.IL-4 recombinant retrovirus were 63.5% and 59.1%, respectively (Fig. 3*B*). The transduced $CD4^+$ TCR transgenic populations were then sorted for low, medium, or high levels of GFP reporter expression. Sorted populations were rested in culture and on day 7 postsort cells were restimulated with MBP peptide, and supernatants were collected for analysis of IL-4 expression by ELISA. Supernatants collected from the TCR transgenic $CD4^+$ T cells transduced with vector alone (pGCIRES) or TCR transgenic $CD4^+$ T cells cultured in the absence of recombinant vector (mock) showed no detectable levels of IL-4 protein (Fig. 3*C*). However, protein levels of IL-4 in pGCIRES.IL-4 low, medium, and high populations directly correlated with GFP expression levels (Fig. 3, *C* and *D*). Subsequent analysis of low, medium, and high expressing GFP populations revealed that the GFP reporter phenotype maintained the defined level of fluorescence over time (data not shown). Importantly, these studies demonstrated a linear

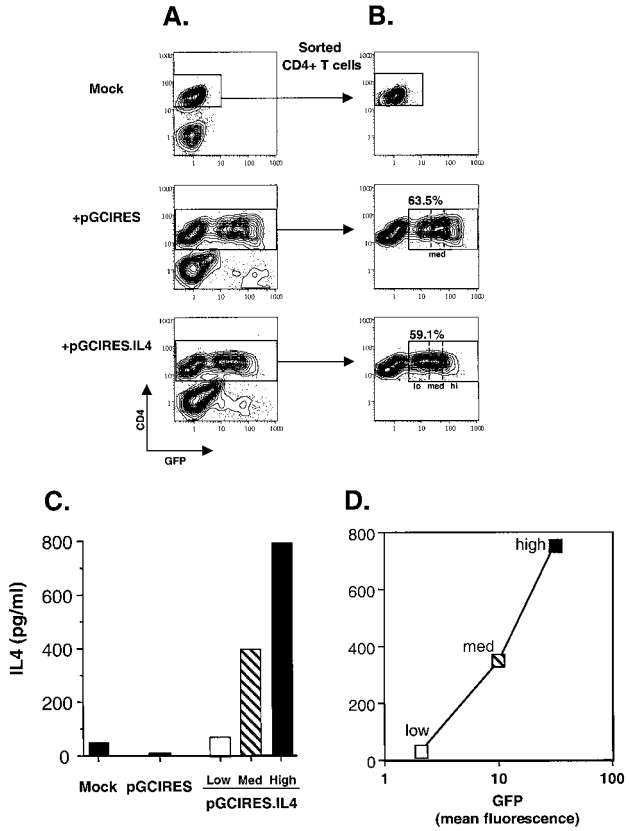

FIGURE 3. A linear relationship exists between GFP reporter expression and upstream gene expression in CD4$^+$ T cells transduced with pGCIRES recombinant retrovirus. *A,* Splenocytes from MBP TCR transgenic mice were cultured in vitro with Ag (MBP NAc_{1-11}) for 24 h before infection with retroviral supernatants containing recombinant vector (pGCIRES), vector with the IL-4 gene (pGCIRES.IL-4), or no vector (Mock). At 48 h postinfection, splenocytes were stained with CD4-PE and analyzed by flow cytometry for GFP expression as a marker of retroviral transduction. *B,* Boxed regions (bold outlines) represent sorted populations and percentages represent transduction efficiencies (% infected) of the CD4$^+$ T cell population. Populations expressing low (lo), medium (med), or high (hi) levels of GFP fluorescence were sorted and placed in culture (stippled regions). Six days postsort, populations were normalized for cell number and restimulated with MBP peptide and irradiated APC. *C,* Bar graph represents presence of IL-4 protein (pg/ml) from supernatants collected at 48 h and analyzed by ELISA. *D,* Graphic representation of IL-4 protein (pg/ml) plotted as a function of GFP mean fluorescence. Data are representative of four independent experiments.

relationship between GFP expression and upstream gene expression in pGCIRES.IL-4-transduced CD4$^+$ T cells. Thus, the pGCIRES retroviral construct allowed GFP reporter gene expression to be used as a direct measure of the upstream gene expression in transduced murine CD4$^+$ T cells.

Retroviral transduction of primed Ag-specific CD4$^+$ T cells

To demonstrate that rare populations of "conventional" Ag-specific CD4$^+$ T cells in primed lymph nodes could be targeted for retroviral transduction, we used DBA/2 mice primed in vivo with SWM peptide 110–121. At 8–10 days postimmunization, draining lymph node and spleen cells were harvested and cultured in vitro with or without the immunizing Ag (SWM). Aliquots of cells were removed from culture each day for 7 days and analyzed for proliferation. Peak proliferation, as demonstrated by incorporation of tritiated thymidine, occurred in spleen and lymph node cultures on days 3 and 4 of stimulation in vitro (data not shown).

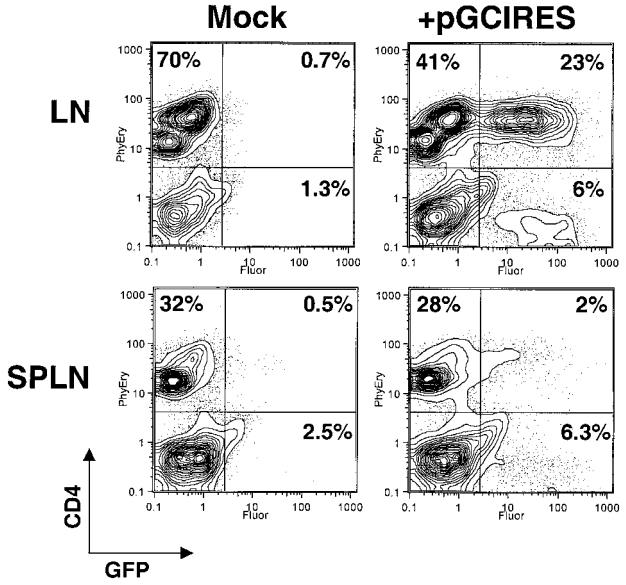

FIGURE 4. Targeting populations of primed murine Ag-reactive CD4$^+$ T lymphocytes using retroviral transduction. Lymph node cells (LN) or spleen cells (SPLN) from DBA/2 mice immunized with SWM were cultured in vitro with SWM peptide, then infected using retroviral supernatant containing either no vector (Mock) or retroviral vector (pGCIRES). At 48 h postinfection, cells were stained with CD4-PE and analyzed by flow cytometry using GFP expression as a marker of retroviral transduction. Percentages represent frequency of cells within each quadrant. Data are representative of four independent experiments performed in triplicate.

In an attempt to capture Ag-reactive cells from primed mice, draining inguinal lymph node cells from SWM-immunized mice were activated in vitro with immunizing peptide, infected with retrovirus, and administered exogenous IL-2 to induce T cell cycling to facilitate retroviral integration following Ag stimulation in vitro. As a control, spleen cells from the immunized mice were infected with retrovirus in parallel and represented a nondraining lymphoid population that would be expected to contain lower frequencies of Ag-reactive cells. Following infection with pGCIRES recombinant retrovirus, lymph node and spleen cell populations were analyzed for transduction using flow cytometry for the evaluation of CD4$^+$ T cells expressing the GFP reporter protein. In a representative experiment, 23% of the total Ag-activated lymph node population, which represents 36% of total CD4$^+$ T cells, was transduced following a 16-h exposure to pGCIRES recombinant retrovirus as demonstrated by GFP reporter expression (Fig. 4). However, only 2% of the Ag-activated cells from the spleen cell population (7% of total CD4$^+$ T cells) were transduced. The transduced non-CD4$^+$ T cells represent cycling B cells in response to exogenously added IL-2 (data not shown). Cells from SWM-stimulated cultures transduced with retroviral supernatant lacking vector DNA (mock) demonstrated only background levels of GFP expression ($<$1%) (Fig. 4). Lymph node or spleen cells from primed mice exposed to pGCIRES recombinant retrovirus and exogenous IL-2, in the absence of the immunizing Ag in vitro, were not infected above background levels (data not shown). Using this model of retroviral infection, we demonstrated transduction efficiencies that were higher than the expected number of Ag-specific CD4$^+$ T cells postulated to exist in draining lymphoid organs (1:5) by limiting dilution studies performed in other animal model systems (32). In subsequent experiments not shown here, it was revealed that the SWM-reactive Ag-specific T cell population (Vβ8$^+$) accounted for ~20% of the transduced CD4$^+$ T cell population with the remainder of the transduced population most

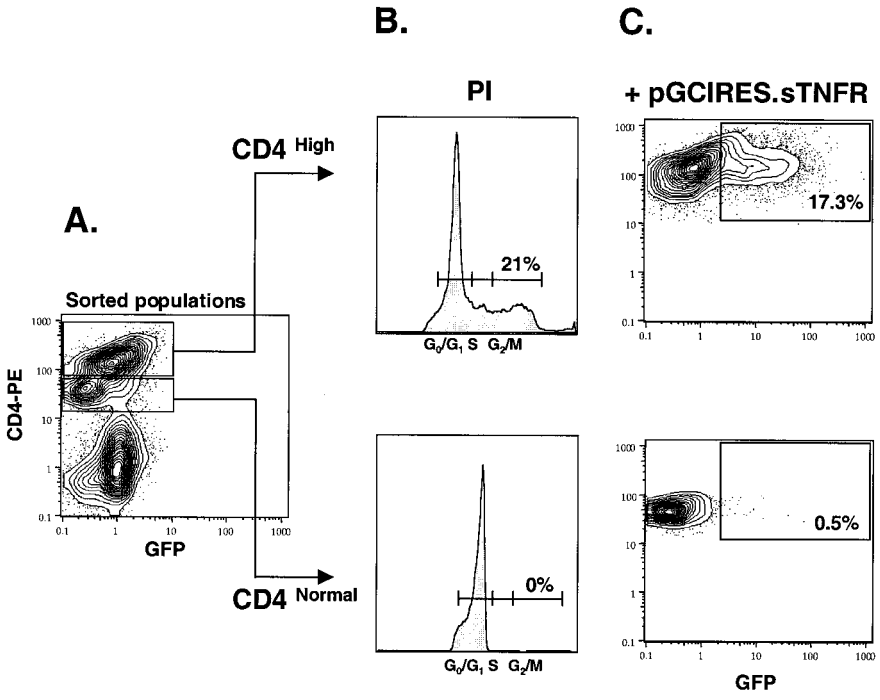

FIGURE 5. Preferential transduction of murine $CD4^{high}$ T cells. *A*, Lymph node cells from DBA/2 mice immunized with SWM peptide were cultured in vitro with SWM peptide for 3 days, and then analyzed for CD4-PE expression. $CD4^{normal}$ and $CD4^{high}$ populations were sorted using flow cytometry as indicated by boxed outlines. *B*, DNA histograms represent the $CD4^{normal}$ and $CD4^{high}$ T cell populations stained using PI before infection with recombinant retrovirus. Horizontal lines are drawn to mark the cell cycle phases: G_0/G_1, S, and G_2/M. The percentage of actively dividing cells, as represented by cells in G_2/M, was 21% in the $CD4^{high}$ population and 0% in the $CD4^{normal}$ population. *C*, Following a 2-h exposure to recombinant retrovirus (+pGCIRES.sTNFR), the CD4 subpopulations were screened using GFP reporter expression as a measure of cell transduction. Transduced populations of the $CD4^{high}$ T cells (17.3%) and the $CD4^{normal}$ T cells (<0.5%) are indicated by box outlines. One representative experiment of three is shown.

likely representative of "adjuvant-activated," possibly purified protein derivative (PPD)-reactive T cells activated in vivo during the priming immunization with CFA and captured in vitro by the addition of IL-2 during retroviral transduction. Most importantly, these studies demonstrated that retroviral infection can be used to target rare populations of conventional nontransgenic, Ag-reactive $CD4^+$ T cells following a single exposure to pGCIRES recombinant retrovirus.

Preferential transduction of $CD4^{high}$ T lymphocytes

Previous studies from our laboratory have demonstrated that Ag-specific, proliferating T cells exhibit a $CD4^{high}$ phenotype (28). We hypothesized that transduction of Ag-specific T cells should preferentially correlate with the $CD4^{high}$ phenotype. Initial evidence for the preferential infection of $CD4^{high}$ T cells was evident upon observation of transduced populations following exposure to recombinant retrovirus. As represented in Fig. 4, lymph node populations stained with Abs to CD4 demonstrate two distinct populations, normal and high, with the $CD4^{high}$-staining cells exhibiting preferential expression of GFP. Therefore, to test the hypothesis that Ag-specific, proliferating T cells exhibiting a $CD4^{high}$ phenotype are preferentially targeted for retroviral transduction, groups of DBA/2 mice were immunized with SWM peptide and draining lymph node cells were harvested and cultured with immunizing Ag in vitro. Following 3 days of in vitro stimulation, cultures were stained with Abs to CD4 and sorted using flow cytometry into two distinct groups, $CD4^{normal}$ and $CD4^{high}$ (Fig. 5*A*). Following cell sorting, $CD4^{normal}$ and $CD4^{high}$ subpopulations were stained with PI to capture the cell cycle profile of sorted populations before retroviral infection. DNA content was analyzed by flow cytometry, and the percentages of $CD4^+$ T cells that were nondividing (G_0/G_1), undergoing DNA synthesis (S) or dividing (G_2/M) were determined (Fig. 5*B*). Only the $CD4^{high}$ subpopulation contained actively dividing cells, represented by a population in G_2/M. In contrast, the $CD4^{normal}$ subpopulation contained no actively dividing cells and the majority of cells were in G_0/G_1. Sorted populations were then exposed to pGCIRES.sTNFR recombinant retrovirus for a decreased infection time of 2 h to allow for the transduction of populations with known cell cycle profiles. Transduction efficiencies of the sorted $CD4^+$ populations were determined by expression of GFP using flow cytometry (Fig. 5*C*). Experiments demonstrated in Fig. 5 revealed that only the $CD4^{high}$ population contained transduced cells and that the number of transduced T cells (17.3%) correlated with the frequency of cells in G_2/M (21%). Conversely, cells from the $CD4^{normal}$ subpopulation contained no transduced T cells (<0.5%) and no detectable proliferating cells (0%). Peak transduction efficiencies were routinely observed when $CD4^{high}$ T cells from immunized mice were exposed to recombinant retrovirus 3–4 days following Ag stimulation in vitro which correlated with peak T cell proliferation profiles exhibited in this model system (data not shown). Transduction efficiencies of $CD4^{normal}$ T cells were negligible at all time periods tested (days 1–7 post-Ag stimulation) (data not shown). These experiments demonstrated that the $CD4^{high}$ phenotype can be used as a marker of Ag reactivity in T cells and that transduction was efficient, but limited to $CD4^{high}$ T cells that were in transit through the M phase at the time of infection. Thus, proliferating, Ag-specific cells can be targeted by infection with retrovirus and isolated by expression of the GFP marker protein.

Transduction of $CD4^{high}$ T cells correlates with mitosis

The relationship between transduction efficiency and cells in G_2/M was consistent in replicate experiments and confirms our previous report which demonstrated that the $CD4^{high}$ population contained all of the Ag-reactive $CD4^+$ T cells (28). To further demonstrate the influence of cell cycle on Ag-specific $CD4^+$ T cell transduction, SWM-specific $CD4^{high}$ T cells were sorted from lymph node cell cultures following 3 days of stimulation in vitro and grown overnight in the presence of IL-2 to induce T cell cycling. $CD4^{high}$ T cells were then stained with a Hoechst vital DNA stain (Hoechst 33342) and analyzed using flow cytometry for cell cycle distribution (G_0/G_1, S+G_2/M, and M subpopulations) according to DNA content (boxed regions, Fig. 6*A*). As expected, the addition of IL-2 increased the number of cycling cells as evidenced by a striking increase in the number of cells cycling through M phase (Fig. 5*B* vs Fig. 6*A*). Viable cell cycle populations were then separated into

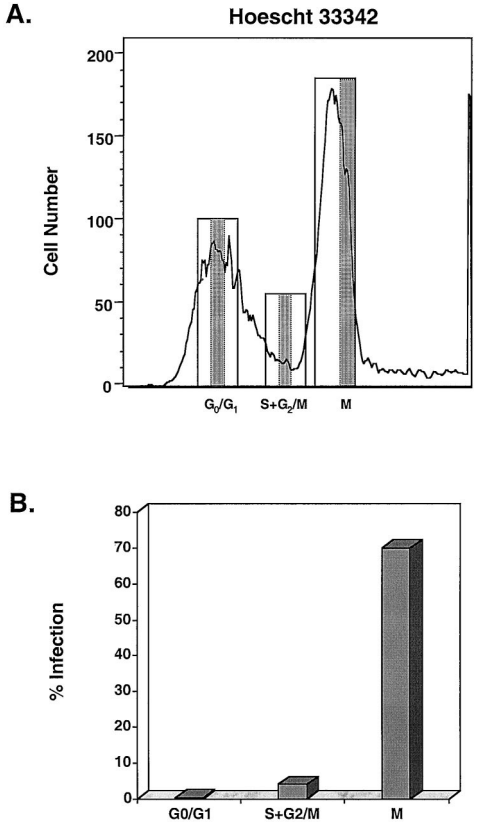

FIGURE 6. Transduction of murine CD4^{high} T cells is cell cycle dependent. SWM-specific CD4^{high} T cells described in Fig. 5 were cultured in vitro overnight with IL-2. *A*, CD4^{high} T cells were then stained with Hoescht 33342 and separated into fractions enriched for cells in G_0/G_1, S, G_2/M, or M phase. Outlines indicate cell cycle phase and shaded areas represent the cell fractions sorted. Cell cycle distribution is plotted on a linear scale. *B*, Sorted CD4$^+$ T cell fractions were exposed to pGCIRES.sTNFR recombinant retrovirus and transduction efficiencies are shown as the percent of GFP$^+$ T cells (% infection) within each cell fraction. Infection percentages for each cell cycle phase are represented by the mean of duplicate samples.

fractions enriched for cells in G_0/G_1, S+G_2/M, or M, and exposed to recombinant retrovirus. For the G_0/G_1 and S+G_2/M fractions, cells were sorted from the central third of their respective staining gates, whereas cells sorted for the M fraction originated from the distal third of the staining gate (shaded regions in Fig. 6*A*). As expected, using GFP expression as a measure of transduction, we found preferential (almost exclusive) retroviral infection of cells fractionated from the M phase subpopulation (73%). None of the cells from the G_0/G_1 phase were productively infected (<0.5%), and only a small percentage of cells from the S+G_2/M phase were infected (<4%) and most likely represented contaminating G_2/M cells within the sorted fraction (Fig. 6*B*). Taken together, these analyses confirmed that efficient retroviral-mediated transduction of CD4^{high} T cells occurred only when cells were actively dividing and in the M phase of the cell cycle.

Discussion

Current therapies for autoimmune diseases include potent and nonspecific immunosuppressive regimens that can lead to systemic toxicity and increased risk for infections and malignancies (33). One difficulty in treating autoimmune diseases is that autoimmune diseases are multifocal (18). However, disease pathology is, in general, localized to specific inflamed tissues. Because autoimmune diseases are the product of transient and localized inflammation, site-specific delivery of regulatory proteins by trafficking autoantigen-specific T lymphocytes may provide a novel method of gene therapy in autoimmunity. Although the identification of immune mechanisms that induce or perpetuate autoimmune disease is not clear, it has been established that autoantigens trigger or perpetuate autoimmunity by stimulation of T lymphocytes within the inflamed tissue. Results presented here demonstrate that Ag-reactive CD4$^+$ T cells can be targeted, ex vivo, for retroviral transduction with therapeutic or anti-inflammatory ("regulatory") molecules for potential application in therapy of autoimmune diseases.

One drawback to retroviral-mediated gene therapy has been the inability to transduce nondividing cells by retroviruses. However, autoantigen-reactive CD4$^+$ T cells proliferate and therefore provide a population suitable for retroviral transduction and for the potential targeted delivery of regulatory (or therapeutic) proteins. In most cell-mediated autoimmune diseases, inflammatory Th1 type T cells reside in the inflamed lesions. To control this inflammation, it is necessary to obtain T cells that can traffic to the lesions and regulate the inflammatory cytokines. To achieve this goal, a robust system of retroviral transduction that would transduce autoantigen-responsive CD4$^+$ T cells was required.

It has been established that oncoretroviruses have the ability to infect dividing cells; therefore, polyclonal activation is generally used as a means to induce proliferation. Data presented here demonstrate that polyclonal activation, before retroviral infection, allowed only transient and nonspecific transduction of multiple murine lymphoid cell lineages, including CD4$^+$ T cells. However, for applications of T cell-based gene therapy, it was only necessary for Ag-specific CD4$^+$ T cell populations to be targeted for retroviral transduction and that transduction of the targeted CD4$^+$ T cells be stable over time. As a model system, we first used TCR transgenic mice containing a population of CD4$^+$ T cells specific for the MBP autoantigen (NAc_{1-11}). CD4$^+$ T cells isolated from MBP-specific TCR transgenic mice allowed us to evaluate the ability of our retroviral vectors to specifically target Ag-activated CD4$^+$ T cells in vitro.

Previous barriers in the application of gene therapy to mouse models of autoimmune diseases have included low proviral integration frequency in immune cells, proviral promoter shutdown, and inadequate isolation and expansion of transduced immunoregulatory cells. Studies presented here demonstrated that Ag-activated CD4$^+$ T cells could be transduced by pGCIRES recombinant retrovirus after stimulation in vitro with specific peptide. Using MBP-TCR transgenic mice, transduced Ag-specific CD4$^+$ T cells exhibited robust infection frequencies (>50%) and stable transgene expression. Transduction using pGCIRES recombinant retrovirus was used to generate MBP-reactive CD4$^+$ T cell lines that demonstrated stable expression of transgenes following four rounds of restimulation with Ag and APC (>40 days). Transgene expression appeared to follow the activation profile of the T cell as evidenced by peak transgene expression 2–3 days following each round of restimulation. Additionally, retroviral transduction of recombinant transgenes did not alter the cytokine profile or the cell surface phenotype of resting and/or activated CD4$^+$ T cells (G. L. Costa and J. M. Benson, unpublished results).

An inherent problem of retroviral transduction is that the transduced populations are heterogenous and contain random integration(s) of provirus and thus relatively random expression of the integrated gene product. For use in gene therapy, a regulated and quantifiable gene product is desired. We solved one of these issues

by demonstrating that expression of the marker protein, GFP, exhibited a linear correlation with upstream gene expression. Thus, using this system, it was possible to select the quantity of "regulatory" protein secreted by the transduced T cells based upon GFP expression. Previous retroviral-mediated delivery systems have used antibiotic- or drug-resistance to select transduced cells (34). However, a drawback of drug selection has been the inability to select populations of transduced cells for multiple (or certainly optimal) therapeutic doses of regulatory proteins. Using pGCIRES recombinant retrovirus for transduction of Ag-specific $CD4^+$ T cells will allow expression of the marker protein, GFP, to select for multiple dose or optimal drug delivery.

Ag-specific $CD4^+$ T cells normally exist at very low frequency in naive as well as in memory T cell pools. Limiting dilution studies have established the frequency of Ag-specific T cells in a naive animal's lymph node at ~1 in 50,000–300,000 (35, 36). Only upon restimulation with specific Ag in vivo do $CD4^+$ T cells undergo extensive expansion that results in an increase in cell number of 150-fold. In model systems defining T cell reactivity in actively primed and rechallenged mice, it has been found that frequencies of Ag-reactive T cells are 1 in 5000 (37). Application of retroviral transduction of autoantigen-reactive $CD4^+$ T cells in gene therapy of autoimmunity must include systems capable of targeting these rare populations of Ag-activated T cells.

As a model system for targeting Ag responsive cells in vivo, we used the DBA/2 $CD4^+$ T cell response to SWM (28). In this system, it was previously demonstrated that the T cells with up-regulated cell surface expression of CD4 ($CD4^{high}$) contained the proliferating, Ag-reactive T cells. Upon retroviral infection of Ag-reactivated bulk cultures, containing either lymph node or spleen cells from SWM-immunized mice, we found that the majority of transduced cells were $CD4^{high}$ T cells (Fig. 4). To confirm our preliminary observations that only $CD4^{high}$ T cells (representing the Ag-reactive T cells) were transduced following retroviral infection, we sorted murine T cell populations on levels of CD4 surface expression, and demonstrated that retroviral infection of an enriched population of $CD4^{high}$ T cells resulted in enhanced transduction efficiency. The $CD4^{normal}$ cells (non-Ag reactive) were not transduced above background.

Because retroviruses integrate the chromosomal DNA of actively dividing cells, we used cell cycle analysis to demonstrate a correlation between $CD4^+$ T cells traversing mitosis and retroviral transduction. Only the $CD4^{high}$ T cell population, containing the Ag-activated $CD4^+$ T cells cycling through G_2/M and M, were transduced. We were able to enhance cell cycling and increase cell transduction by the addition of IL-2 to the Ag reactivated T cell cultures. Ag-stimulated $CD4^{high}$ T cells, supplemented with exogenous IL-2 for 24 h before cell cycle analyses, exhibited a dramatic enrichment of cells cycling through mitosis. Although IL-2 was necessary for the optimization of transduction in Ag-stimulated $CD4^+$ T cells, exogenously added IL-2, in the absence of specific Ag, did not support efficient transduction of $CD4^+$ T cells (Fig. 3). The application of retroviral transduction coupled with exogenously added IL-2, which serves to both facilitate retroviral transduction and to expand target Ag-activated T cells, should prove beneficial when attempting to isolate rare populations of autoreactive cells with unknown Ag(s) specificity, as in animal models for human diseases such as multiple sclerosis, diabetes, and rheumatoid arthritis.

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References

1. Holoshitz, J., Y. Naparstek, A. Ben-Nun, P. Marquardt, and I. R. Cohen. 1984. T lymphocyte lines induce autoimmune encephalomyelitis, delayed hypersensitivity and bystander encephalitis or arthritis. *Eur. J. Immunol. 14:729.*
2. Holmdahl, R., V. Malmstrom, and E. Vuorio. 1993. Autoimmune recognition of cartilage collagens. *Ann. Med. 25:251.*
3. Rosenberg, S. A. 1991. Immunotherapy and gene therapy of cancer. *Cancer Res. 51:5074s.*
4. Wels, W., D. Moritz, M. Schmidt, M. Jeschke, N. E. Hynes, and B. Groner. 1995. Biotechnological and gene therapeutic strategies in cancer treatment. *Gene 159:73.*
5. Zamvil, S. S., and L. Steinman. 1990. The T lymphocyte in experimental allergic encephalomyelitis. *Annu. Rev. Immunol. 8:579.*
6. Roberts, M. R., L. Qin, D. Zhang, D. H. Smith, A. C. Tran, T. J. Dull, J. E. Groopman, D. J. Capon, R. A. Byrn, and M. H. Finer. 1994. Targeting of human immunodeficiency virus-infected cells by $CD8^+$ T lymphocytes armed with universal T-cell receptors. *Blood 84:2878.*
7. Liblau, R. S., S. M. Singer, and H. O. McDevitt. 1995. Th1 and Th2 $CD4^+$ T cells in the pathogenesis of organ-specific autoimmune diseases. *Immunol. Today 16:34.*
8. Dolhain, R. J., A. N. van der Heiden, N. T. ter Haar, F. C. Breedveld, and A. M. Miltenburg. 1996. Shift toward T lymphocytes with a T helper 1 cytokine-secretion profile in the joints of patients with rheumatoid arthritis. *Arthritis Rheum. 39:1961.*
9. Horsfall, A. C., D. M. Butler, L. Marinova, P. J. Warden, R. O. Williams, R. N. Maini, and M. Feldmann. 1997. Suppression of collagen-induced arthritis by continuous administration of IL-4. *J. Immunol. 159:5687.*
10. Racke, M. K., A. Bonomo, D. E. Scott, B. Cannella, A. Levine, C. S. Raine, E. M. Shevach, and M. Rocken. 1994. Cytokine-induced immune deviation as a therapy for inflammatory autoimmune disease. *J. Exp. Med. 180:1961.*
11. Tominaga, Y., M. Nagata, H. Yasuda, N. Okamoto, K. Arisawa, H. Moriyama, M. Miki, K. Yokono, and M. Kasuga. 1998. Administration of IL-4 prevents autoimmune diabetes but enhances pancreatic insulitis in NOD mice. *Clin. Immunol. Immunopathol. 86:209.*
12. Abbas, A. K., K. M. Murphy, and A. Sher. 1996. Functional diversity of helper T lymphocytes. *Nature 383:787.*
13. Mosmann, T. R., and S. Sad. 1996. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol. Today 17:138.*
14. Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol. 136:2348.*
15. O'Garra, A. 1998. Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity 8:275.*
16. Kumar, V., and E. Sercarz. 1998. Induction or protection from experimental autoimmune encephalomyelitis depends on the cytokine secretion profile of TCR peptide-specific regulatory CD4 T cells. *J. Immunol. 161:6585.*
17. King, C., and N. Sarvetnick. 1997. Organ-specific autoimmunity. *Curr. Opin. Immunol. 9:863.*
18. Mathisen, P. M., and V. K. Tuohy. 1998. Gene therapy in the treatment of autoimmune disease. *Immunol. Today 19:103.*
19. Kramer, R., Y. Zhang, J. Gehrmann, R. Gold, H. Thoenen, and H. Wekerle. 1995. Gene transfer through the blood-nerve barrier: NGF-engineered neuritogenic T lymphocytes attenuate experimental autoimmune neuritis. *Nat. Med. 1:1162.*
20. Bessis, N., M. C. Boissier, P. Ferrara, T. Blankenstein, D. Fradelizi, and C. Fournier. 1996. Attenuation of collagen-induced arthritis in mice by treatment with vector cells engineered to secrete interleukin-13. *Eur. J. Immunol. 26:2399.*
21. Evans, C. H., P. D. Robbins, S. C. Ghivizzani, J. H. Herndon, R. Kang, A. B. Bahnson, J. A. Barranger, E. M. Elders, S. Gay, M. M. Tomaino, et al. 1996. Clinical trial to assess the safety, feasibility, and efficacy of transferring a potentially anti-arthritic cytokine gene to human joints with rheumatoid arthritis. *Hum. Gene Ther. 7:1261.*
22. Shaw, M. K., J. B. Lorens, A. Dhawan, R. DalCanto, H. Y. Tse, A. B. Tran, C. Bonpane, S. L. Eswaran, S. Brocke, N. Sarvetnick, et al. 1997. Local delivery of interleukin 4 by retrovirus-transduced T lymphocytes ameliorates experimental autoimmune encephalomyelitis. *J. Exp. Med. 185:1711.*
23. Dal Canto, R. A., M. K. Shaw, G. P. Nolan, L. Steinman, and C. G. Fathman. 1999. Local delivery of TNF by retrovirus-transduced T lymphocytes exacerbates experimental autoimmune encephalomyelitis. *Clin. Immunol. 90:10.*
24. Chen, L. Z., G. M. Hochwald, C. Huang, G. Dakin, H. Tao, C. Cheng, W. J. Simmons, G. Dranoff, and G. J. Thorbecke. 1998. Gene therapy in allergic encephalomyelitis using myelin basic protein-specific T cells engineered to express latent transforming growth factor-β1. *Proc. Natl. Acad. Sci. USA 95:12516.*
25. Lafaille, J. J., K. Nagashima, M. Katsuki, and S. Tonegawa. 1994. High incidence of spontaneous autoimmune encephalomyelitis in immunodeficient anti-myelin basic protein T cell receptor transgenic mice. *Cell 78:399.*
26. Pear, W. S., G. P. Nolan, M. L. Scott, and D. Baltimore. 1993. Production of high-titer helper-free retroviruses by transient transfection. *Proc. Natl. Acad. Sci. USA 90:8392.*
27. Kinsella, T. M., and G. P. Nolan. 1996. Episomal vectors rapidly and stably produce high-titer recombinant retrovirus. *Hum. Gene Ther. 7:1405.*

28. Ridgway, W., M. Fasso, and C. G. Fathman. 1998. Following antigen challenge, T cells up-regulate cell surface expression of CD4 in vitro and in vivo. *J. Immunol. 161:714.*
29. Evstafieva, A. G., T. Y. Ugarova, B. K. Chernov, and I. N. Shatsky. 1991. A complex RNA sequence determines the internal initiation of encephalomyocarditis virus RNA translation. *Nucleic Acids Res. 19:665.*
30. Davies, M. V., and R. J. Kaufman. 1992. The sequence context of the initiation codon in the encephalomyocarditis virus leader modulates efficiency of internal translation initiation. *J. Virol. 66:1924.*
31. Karavanas, G., M. Marin, B. Salmons, W. H. Gunzburg, and M. Piechaczyk. 1998. Cell targeting by murine retroviral vectors. *Crit. Rev. Oncol. Hematol. 28:7.*
32. Weyand, C. M., J. Goronzy, M. J. Dallman, and C. G. Fathman. 1986. Administration of recombinant interleukin 2 in vivo induces a polyclonal IgM response. *J. Exp. Med. 163:1607.*
33. Feldmann, M., F. M. Brennan, and R. Maini. 1998. Cytokines in autoimmune disorders. *Int. Rev. Immunol. 17:217.*
34. Flugel, A., M. Willem, T. Berkowicz, and H. Wekerle. 1999. Gene transfer into $CD4^+$ T lymphocytes: green fluorescent protein- engineered, encephalitogenic T cells illuminate brain autoimmune responses. *Nat. Med. 5:843.*
35. Ford, D., and D. Burger. 1983. Precursor frequency of antigen-specific T cells: effects of sensitization in vivo and in vitro. *Cell. Immunol. 79:334.*
36. Gebel, H. M., J. R. Scott, C. A. Parvin, and G. E. Rodey. 1983. In vitro immunization to KLH. II. Limiting dilution analysis of antigen-reactive cells in primary and secondary culture. *J. Immunol. 130:29.*
37. Kojima, M., K. B. Cease, G. K. Buckenmeyer, and J. A. Berzofsky. 1988. Limiting dilution comparison of the repertoires of high and low responder MHC-restricted T cells. *J. Exp. Med. 167:1100.*