**In vivo** Imaging and Quantitation of Adoptively Transferred Human Antigen-Specific T Cells Transduced to Express a Human Norepinephrine Transporter Gene

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**Abstract**
Sequential imaging of genetically marked effector cells after adoptive transfer in vivo has greatly enhanced analyses of their biodistribution, growth, and activity both in animal models and in clinical trials of cellular immunotherapy. However, the immunogenicity of cells expressing xenogeneic reporter constructs limits their survival and clinical utility. To address this limitation, we have evaluated a human norepinephrine transporter (hNET) permitting imaging of transduced cells in vivo with a previously approved clinical grade radiolabeled probe, metaiodobenzylguanidine (MIBG). The hNET gene cDNA was cloned from the SK-N-SH cell line and inserted into a bicistronic retroviral vector also encoding green fluorescent protein. Following transfection, human EBV-specific T lymphocytes were fully functional in vitro and also selectively accumulated [123I]MIBG. In nonobese diabetic/severe combined immunodeficient mice bearing human EBV lymphoma xenografts, as few as 10⁴ transduced T cells injected into the tumors could be imaged by single-photon emission computed tomography (SPECT) or positron emission tomography (PET) after i.v. infusion of [123I]MIBG or [124I]MIBG, respectively. When hNET+ EBV-specific T cells were infused i.v., their migration and specific accumulation in EBV+ tumors expressing their restricting HLA allele could be imaged by SPECT or PET over 28 days. Image intensity was closely correlated with the number of T cells accumulated in targeted tumors. The use of two reporter probes (MIBG and 2-deoxy-2-fluoro-β-D-arabinofuranosyl-5-iodouracil) permitted independent contemporaneous tracking of two distinct EBV-specific T-cell subpopulations expressing different reporter genes (hNET–CD4+ T cells and HSV-TK-CD8+ T cells) in the same animal using three-dimensional nuclear modalities (SPECT and PET). The hNET-based system described may thus have significant potential as a nonimmunogenic reporter for extended repeated quantitative in vivo imaging of transduced cells in man. [Cancer Res 2007;67(24):11959–69]

**Introduction**
Evaluations of biological therapies targeting human malignancies increasingly depend on thorough monitoring of the biodistribution and ultimate disposition of the therapeutic agents following their introduction into the organism and throughout the course of their treatment effects. Molecular gene imaging offers a convenient tool for noninvasive observation of the biological processes in live subjects and also in real time (1). However, effective clinical translation has been subject to several limitations (2), particularly the immunogenicity of the reporter gene product. Use of a protein of human origin as the reporter may circumvent this problem. Several human reporter genes have been developed, including human dopamine D2 receptor (hDDR2), human sodium iodine symporter (hNIS), and human somatostatin receptor subtype 2 (hSSTR2; refs. 3–7). Another is the human norepinephrine transporter (hNET; ref. 8), a protein involved in the transport of norepinephrine analogues into cells. Its properties have been used as a reporter: (a) it is a human protein that should minimize its immunogenicity; (b) a radiolabeled probe for imaging hNET expression is currently used in the clinic; (c) this probe, MIBG, can be radiolabeled with 123I or 131I for single-photon emission computed tomography (SPECT) and γ-camera imaging and with 124I for positron emission tomography (PET) imaging.

In this study, we have examined the feasibility of using the hNET-based reporter system for noninvasive in vivo imaging of human antigen-specific CTLs in a murine model of adoptive immune cell therapy of human EBV-associated lymphoma with [123I]MIBG and SPECT or [124I]MIBG and PET. We also evaluated the potential of hNET, when used in combination with a second clinically applicable reporter, for dual-modality imaging to monitor two separate lymphocyte subpopulations in vivo.

**Materials and Methods**
Cloning cDNA for hNET and construction of retroviral vectors for directing constitutive expression of hNET. The hNET gene was cloned as described previously (8). Specific primers (GeneLink) were designed for the 3’ and 5’ ends of the cDNA sequences of hNET, described in the sequence database (NM_001043, M65105). hNET cDNAs were amplified by reverse transcription-PCR from the neuroblastoma cell line SK-N-SH (American Type Culture Collection; ref. 8). After separation in agarose gel, a band corresponding to the molecular weight of hNET was extracted, purified, and used for vector production according to techniques described previously (8). Briefly, cDNA fragments were cloned into the pGEM-TEasy vector (Promega) and checked using standard T7 and SP6 primers. The product was ligated with pQCXIX (Clontech). An hNET-IRES fragment was obtained from the resulting pQCXNERTX plasmid using BamHI and XhoI endonucleases (New England Biolabs) and cloned into pLEGFP-N1 backbone using the BglII and XhoI insertion sites. The precise vector used for transduction,

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which is pHNetIGFP, is produced by insertion of hNET-IREs fragment into pLEGFP-Nq plasmid (Clontech). The DNA obtained was used to transfect a packaging cell line, H29, for subsequent transduction of the retroviral producer cell line, PG-13 (Clontech).

**Cell line transduction, selection, and testing.** The technique used for transduction of the T cells is based on that described by Pollok et al. (13) as modified for transduction of T cells specifically sensitized with autologous EBV-transformed B cells (EBV-BLCL; refs. 14, 15). Briefly, aliquots of 2 mL/well of isolated T cells prestimulated with autologous irradiated EBV-BLCL for 8 days were cultured for 8 h at a concentration of 1 × 10^6/mL in nontissue culture six-well plates (Costar) coated with 15 μg/cm² retictron (recombinant human fibronectin fragment CH-296, Takara Bio, Inc.) together with a 35-fold concentrate of cell-free retroviral vector-containing supernatant from the PG-13 cells at a final concentration of 10^6 infectious viral particles/mL (multiplicity of infection = 10; as determined by terminal dilution transfection of Jurkat cells) and 8 μg/mL polybrene (American Bioanalytical). After washing and culturing for 12 h, the transduction was repeated. Thereafter, the washed cells were incubated for 3 days in Yssel’s medium supplemented with 5% heat-inactivated human serum and 10 μL/mL of recombinant human interleukin-2 (IL-2; Chiron, Inc.) at a concentration of 0.6 × 10^6 T cells/mL. The transduced T cells were then analyzed and sorted in a FACS Calibur sterile sorter (Becton Dickinson).

Separate aliquots of T cells from the same CTL line were simultaneously transduced with a previously described TK-eGFP vector following the same protocol for transduction and sorted by expression of green fluorescent protein (GFP). In separate experiments, subsets of CD4+ and CD8+ EBV-specific T lymphocytes were fractionated from the parental parental T cells by fluorescence-activated cell sorting (FACS) of cells stained with fluorescent anti-CD4 or anti-CD8 antibodies (Becton Dickinson), separately transduced with hNETIGFP and Tk-eGFP vectors, respectively, and selected by FACS based on GFP expression.

Expression of hNET was assessed by FACS staining with mouse anti-human NET antibodies (Mab Technologies, Inc.) and secondary goat anti-mouse phycoerythrin (PE)-conjugated antibodies (Becton Dickinson). The level and the rate of transporter activity were evaluated using a standard in vitro uptake assay (2, 14) by measuring the accumulation of specific ([123I]MIBG) and nonspecific ([124I]2-deoxy-2-fluoro-D-glucose) radiotracers in hNET-transduced cells (CTL-hNETIGFP) and compared with the uptake of these radiotracers in TK-eGFP-transduced T cells (CTL-TKGFP) isolated from the same EBV-CTL.

To assess the influence of the new reporter gene on transduced T cells, the proliferation and functional activity of CTL-hNETIGFP were compared with those of unmodified CTL or CTL-TKGFP of the same origin. Proliferation was assessed by monitoring the total number of viable cells in the CTL cultures at specified intervals. To assess the influence of specific radiolabels on the EBV-specific cytotoxicity of CTL-hNETIGFP, CTL-TG, and unmodified CTL, each cell type was preincubated for 3 h with 0.1 μCi/mL of [123I]MIBG, [123I]MIBG, [124I]FAU, or MIBG alone. EBV-specific cytotoxicity and HLA restriction were defined in standard 51Cr release assays against a panel of EBV+ and EBV− targets sharing or not sharing specific HLA alleles with the T-cell donor as described previously (14) and compared with the responses of the same T cells incubated with medium alone.

**Human EBV lymphoma xenograft model.** For the in vivo imaging studies, we used a model of adoptive cell therapy previously developed in our laboratory (14, 16). Briefly, as required for specific experiments, groups of at least three and up to five nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice received one or four anatomically separated s.c. inoculations of different human EBV-BLCL (BLCL) or human leukemic cells, varying in expression of EBV and/or HLA alleles (3 × 10^6 to 5 × 10^6 cells per injection). Sorted populations of CTL-hNETIGFP or CTL-TKGFP were given either intratumorally or i.v. when measurable tumor burden was detected at all sites. At specified times thereafter, the animals received i.v. infusions of the specific radiolabeled probes and were subsequently imaged by PET or SPECT.

PET imaging was performed on a MicroPET-IIIr (Concorde) scanner using 15-min list-mode acquisition of a single static frame (energy range, 420–580 keV). Images were obtained weekly 4 or 24 h after administration of 200 μCi of the [124I]labeled radiotracers. To compare MIBG imaging by PET and SPECT, a combination of 200 μCi [123I]MIBG and 500 μCi [124I]MIBG was given 4 h before sequential PET and SPECT imaging sessions, respectively. Each PET image was reconstructed using a filtered back projection algorithm. Coronal sections were used to draw regions of interest (ROIs) and quantitatively evaluate the radiotracer accumulation.

SPECT images were acquired for 30 min, beginning 4.5 h after administration of 500 μCi [123I]MIBG or 500 μCi [124I]MIBG and the longer exposure time used for image acquisition were selected to yield images comparable with those generated in PET scans evaluating accumulations of [131I]labeled MIBG or FIAU. A structural image of the animal using CT scan was obtained with 128 frames immediately following the SPECT acquisition. Image reconstruction was performed using filtered back projections applying a Butterworth 50 filter. CT scans were reconstructed using Cobra software. Coregistration of CT and SPECT images was performed on X-SPECT with the vendor’s software.

**Statistical analysis.** The coefficient of determination (R-squared) was used to assess the fit of the regression line to the data points. The R-squared statistic ranges between zero and one, where a value of one indicates a perfect fit of the regression line to the data and a value of zero represents that a linear relationship does not exist between the two variables under study. A P value was generated to provide a level of evidence that a linear relationship exists between each pair of variables. For paired comparisons of samples, the χ^2 and Fisher’s exact tests were applied.

**Results**

**Characterization of EBV-CTL transduced to express hNET reporter gene system.** Sequencing analysis showed identity of 5′ and 3′ ends of cloned hNET with the published National Center for Biotechnology Information sequence (M65105). Following pLhNET-IGFP retroviral vector transfection, >25% of primary EBV-CTL expressed hNET and GFP, which were then selected by GFP expression to 98% purity (Fig. 1A).

[123I]MIBG accumulation in hNET-transduced primary human antigen-specific T lymphocytes in vitro.** The influx constant K1 was 1.8 ± 0.5 min⁻¹/g and the steady-state accumulation level, K1/K2, was 58.5 ± 6.8 mL/g. The mean value for the accumulation observed at 60 min in three repeated tests was 58.5 ± 3.0 mL/g. This level of in vitro activity of the hNET reporter was slightly higher than that of HSV-TK, as measured by accumulation of [124I]FAU, detected in CTL-TKGFP. Therefore, we hypothesized that in vivo MIBG uptake of CTL-hNETIGFP would be sufficient to yield images at least comparable with those achievable using [124I]FAU to image HSV-TK–transduced cells (Fig. 1B; ref. 14).

CTL-hNETIGFP proliferated in response to antigen-specific stimulation (Fig. 1D) at levels similar to those detected in unmodified CTL or CTL-TKGFP from the same EBV-specific T-cell line. They also exhibited comparable HLA-restricted EBV-specific cytotoxicity (data not shown). The level of EBV-specific cytotoxicity of CTL-hNETIGFP was not affected by preincubation of the T cells with unlabeled MIBG or MIBG labeled with either [123I] or [124I] when compared with CTL-TKGFP or nontransduced EBV-specific T cells from the same line (Fig. 1C).

**Sensitivity of PET and SPECT for imaging hNET+ T cells in vivo.** We initially wished to determine the number of CTL-hNETIGFP detectable in a targeted tumor in vivo. Accordingly, groups of mice, each bearing a 1 cm³ s.c. EBV-BLCL tumor xenograft, received direct intratumoral injections of doses of 10⁴, 10⁵, 10⁶, or 5 × 10⁶ of EBV-specific CTL-hNETIGFP per tumor.
(n = 3 mice/CTL dose) in 100 μL of saline followed by an i.v. infusion of a combined dose of 200 μCi [124I]MIBG and 500 μCi [123I]MIBG. Four hours thereafter, the mice were imaged by PET for 15 min and, subsequently, by SPECT for 30 min.

In these experiments, we also wished to gain an initial assessment of the capacity of PET to image EBV-specific CTL-hNETiGFP accumulating [123I]MIBG when compared with EBV-specific CTL-TKGFP, which, as we previously reported (14), can be imaged based on their accumulation of [124I]FIAU. Accordingly, we treated separate groups of NOD/SCID mice bearing EBV-BLCL tumor xenografts with intratumoral injections of EBV-specific CTL-TKGFP from the same parental T-cell line at doses of 10^4, 10^5, and 10^6 T cells per tumor (n = 3 mice/CTL dose) followed by an i.v. infusion of 200 μCi [124I]FIAU and, 4 h thereafter, imaging by PET.

As shown in Fig. 2A, the minimal dose of CTL-hNETiGFP detected by PET was 10^4 cells. The signal intensity recorded in the tumors by PET and the well count activity detected in the tumors isolated after autopsy (Fig. 2B) correlated closely with the dose of T cells injected (P < 0.001). As shown in Fig. 2C, PET images of mice injected with graded doses of EBV-specific CTL-TKGFP were also able to distinguish tumors injected with as few as 10^4 T cells (Fig. 2C). The dose of CTL-TKGFP inoculated was also significantly correlated with tumor uptake of [124I]FIAU (Fig. 2D). However, the steeper slope of the plot in Fig. 2D suggests that PET imaging of
CTL-TKGFP may be more sensitive for quantitations of T cells accumulated.

Of note, the whole-body PET images obtained within 4 h after i.v. infusion of each radiotracer also showed high level of radioactivity in the gut and urinary tract caused by excretion of the 124I metabolites (Fig. 2A and C). These nonspecific accumulations were detected both in the animals that had received intratumoral injections of T cells and in control animals that had not received injections of T cells. Although these nonspecific accumulations can be avoided by obtaining PET images 24 h after infusion of the radiotracer (additional images not shown; ref. 14), images obtained within 4 h of inoculation of T cells and infusions of the radiotracer were used to estimate the numbers of T cells to minimize inaccuracies potentially caused by local diffusion or systemic redistribution of the intratumorally injected T cells through the circulation or by replication or apoptosis of the T cells after interaction with their targets.

Subsequent X-SPECT images of the injected tumors obtained 4.5 h after the coadministration of 500 μCi/mouse [123I]MIBG and 200 μCi/mouse [124I]MIBG indicated that accumulation of as few as 10^4 hNETiGFP+ T cells could also be imaged by this modality (Fig. 3A). Application of dual-modality imaging with CT overlapping the SPECT image permitted exact localization of the increased radiotracer accumulation area to the tumor. Again, measurements of signal intensity showed a direct correlation between signal intensity and dose of T cells injected in each tumor (P < 0.001; Fig. 3B). As expected, and consistent with our prior studies (14), there was also a close correlation between the amount of radiotracer detected by the γ-spectrometer well counter in the tumors and the dose of hNET+ T cells previously injected into the tumor.

Measurements of accumulation of EBV-specific CTL-hNETiGFP in a target tumor over extended time intervals. In our prior studies, we had shown that accumulations of EBV-specific T cells transduced to express HSV-TK in targeted xenografts could be imaged in vivo over a 2-week period by sequential infusions of [124I]FIAU followed by PET. To assess the utility of hNET as a reporter for long-term monitoring of EBV-specific T cells, EBV-specific CTL-hNETiGFP at doses of 12 to 10^6 CTLs were infused i.v. into five mice bearing a single s.c. tumor xenograft of autologous EBV-BLCL. At 1, 8, and 28 days after T-cell infusion, the animals were injected with 200 μCi/mouse [124I]MIBG. Four hours thereafter, they were imaged by PET.

As shown in Fig. 4A, a progressive increase in the PET signal was detected in the tumors over 28 days. The number of T cells...
accumulated in the targeted tumors was estimated by calculation of the number of T cells in the tumors based on the radiotracer accumulation per dose of T cells plot in Fig. 2B and confirmed by quantitation of the radioactivity in the targeted tumors and by enumeration of the absolute number of GFP+ human T cells in the targeted tumor (Fig. 4B). The initial accumulation of T cells in the tumors, calculated from the PET signal intensity of \[^{124}\text{I}]\text{MIBG}\) activity as described earlier (Fig. 2A), was \(\sim 0.5 \times 10^6\) T cells and increased to \(\sim 6 \times 10^6\) T cells by day 28. As shown in Fig. 4B, there was a direct and significant correlation between cell numbers in each tumor calculated based on well count activity of \[^{124}\text{I}]\text{MIBG}\) in the targeted tumor and the signal accumulation recorded for each tumor by PET imaging \((P < 0.001)\). The number of cells in the tumors calculated based on radioisotope accumulation was also significantly correlated with the number of GFP+ T cells detected in the tumor \((R^2 = 0.73; P = 0.043)\).

**PET and SPECT imaging of targeted accumulations of virus-specific CTL-hNETiGFP in EBV+ tumors over time.** To evaluate the antigen-targeted accumulation of EBV-specific CTL-hNETiGFP over time, we treated a group of five mice, each bearing four distinct anatomically separated EBV+ and EBV− tumor xenografts differing in their HLA genotypes, with i.v. infusions of \(12 \times 10^6\) EBV-specific CTL-hNETiGFP per mouse. On days 1, 8, 21, and 28 thereafter, the mice received a combined infusion of 500 \(\mu\text{Ci}\) \[^{123}\text{I}]\text{MIBG}\) and 200 \(\mu\text{Ci}\) \[^{124}\text{I}]\text{MIBG}\) (days 1, 8, and 28); due to limited availability of \[^{124}\text{I}]\text{MIBG}\), only \[^{123}\text{I}]\text{MIBG}\) was given on day 21. PET and/or SPECT images were obtained 4 and 4.5 h after infusion of the radiolabeled MIBG, respectively. In this experiment, the

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**Figure 3.** SPECT/CT images of \[^{123}\text{I}]\text{MIBG}\) accumulation in targeted tumors following direct intratumoral injection of graded doses of CTL-hNETiGFP. The same mice infused with \[^{124}\text{I}]\text{MIBG}\) and \[^{123}\text{I}]\text{MIBG}\) imaged by PET in Fig. 2 were also imaged by SPECT/CT 4.5 h after radiotracer administration. A, two sets of mice imaged by SPECT/CT with hNET standards. In the two sets, the juxtapositioning of mice injected with different T-cell doses was altered so as to permit image intensity comparisons within the maximal CT reconstruction diameter. Image coregistration was performed using X-SPECT software, combining two sets of axial reconstruction data. To eliminate the signal originating from the downscatter of \[^{124}\text{I}]\text{MIBG}\) in the \[^{123}\text{I}]\text{MIBG}\) SPECT images, we determined the \[^{124}\text{I}]\text{MIBG}\) downscatter coefficient in the \[^{123}\text{I}]\text{MIBG}\) energy window (146-175 keV) of the LumagEM \(\gamma\)-camera of the X-SPECT for serial dilutions of the isotope standards over a broad range of concentrations, including the percent dose per gram range observed in the current study (30-0.001% dose/g) for the fixed dose activity ratio given to the animals, which was 5:2 \[^{123}\text{I}]\text{MIBG}\) to \[^{124}\text{I}]\text{MIBG}\). This coefficient, which, on average, was 11%, was then used in our calculations of ROI. For well count corrections, we used direct decay-corrected count substractions. B, correlation between the dose of CTL-hNETiGFP injected into each tumor and both the accumulated activity detected in the ROI of each tumor by SPECT (solid line) and the amount of radiolabeled \[^{124}\text{I}]\text{MIBG}\) detected in the autopsied tumors (dotted line). Both signal intensity and accumulation of radiolabel were closely correlated with the dose of CTL-hNETiGFP injected \((P < 0.001,\) for both correlations).
EBV-specific CTL-hNETiGFP used contained a predominance of CD8+CD4+ double-positive CD3+ T cells restricted by HLA-A0201 together with CD4+ T cells restricted by HLA-DRB10402. As shown in the SPECT and SPECT-CT fusion images in Fig. 5A as well as the PET images in Fig. 5B, radiolabeled MIBG was selectively and progressively accumulated in the HLA-A0201 EBV+ tumor (T1) from day 1 to day 28. In contrast, accumulations of [123I]MIBG and [124I]MIBG in the HLA-DRB10402 EBV+ tumor (T4) were not

Figure 4. Time course studies of migration of EBV-specific T cells in the in vivo lymphoma model. A, PET images of mice bearing a single s.c. EBV-BLCL xenograft, which received a single i.v. infusion of 12 × 10^6 hNETiGFP+ EBV-specific T cells per mouse (n = 5). At 1, 8, and 28 d after T-cell infusion, each mouse received a dose of 200 μCi/mouse [124I]MIBG and was imaged by PET within 4 h. T cells were detectable in the tumor by day 1 and progressively accumulated from day 1 to day 28. In contrast, accumulations of [123I]MIBG and [124I]MIBG in the HLA-DRB10402 EBV+ tumor (T4) were not...
detected by either SPECT or PET until day 28 after infusion. Quantitations of radioactivity accumulated in the tumors and normal tissues obtained at autopsies on day 28 (Fig. 5C) showed significantly increased uptake of MIBG in the T1 tumor as well as a less striking increase of label in the T4 tumor. In contrast, labeled MIBG did not accumulate in the allogeneic EBV+ (T2) or HLA-A0201 ‘DRB,0402 ‘EBV’ tumor (T3). As expected, uptake of MIBG was increased in the heart and adrenal glands relative to normal tissues. Indeed, the accumulation of labeled MIBG in the T4 tumor detected at day 28 was only moderately greater than that detected in the heart and adrenal glands. Taken together, these studies of EBV-specific CTL-hNETiGFP confirm and extend our prior studies showing selective accumulation of EBV-specific, HLA class I–restricted CD8+ T cells in tumors expressing EBV and the restricting HLA allele (14). Sequential imagings showed the ability of HLA class II–restricted CD4+ T cells to accumulate in tumors expressing EBV and their restricting allele (i.e., DRB,0402) but also showed that limited populations of antigen-specific T cells may not be imaged in targeted tissues until they have expanded to sufficient numbers to permit their detection.

**Dual-modality imaging.** Based on the comparable imaging of CTL-hNETiGFP and CTL-TKGFP, we explored whether these two reporters could be simultaneously used to evaluate the migration of two separate T-cell populations in the same animal. As shown in Fig. 6A, after coinfusion of [124I]FIAU and [123I]MIBG, PET showed selective [124I]FIAU accumulation by CD8+ CTL-TKGFP in the EBV+HLA-A0201 tumor (T1). No signal was detected in the T2 tumor. Thus, PET scan specifically registered signals from HSV-TK+CD8+ T cells accumulating [124I]FIAU but not from CD4+ T cells accumulating [123I]MIBG, reflecting the ability of PET to exclude the low-energy γ-rays emitted by [123I]MIBG. In contrast, although SPECT imaging showed maximal signal in the HLA-DRB,0701 ‘EBV’ tumor (T2) targeted by the CD4+ EBV-specific CTL-hNETiGFP, reflecting their accumulation of [123I]MIBG, low

![Dual-modality imaging](image)

**Figure 5.** Sequential monitoring of EBV-specific HLA-restricted accumulation of CTL-hNETiGFP over 1 mo using [123I]MIBG and SPECT/CT fusion or [124I]MIBG and PET. In this experiment, each of five mice evaluated bore four tumor xenografts: an HLA-A0201 ‘DRB,0402 ‘EBV-BLCL (T1), a fully HLA mismatched EBV-BLCL (T2), an HLA-A0201 ‘DRB,0402 ‘EBV-BLCL (T3), and an HLA-A0201 ‘DRB,0402 ‘EBV-BLCL (T4). CTL-hNETiGFP/mouse containing both HLA-A0201–restricted and HLA-DRB,0402–restricted, EBV-specific T cells was injected i.v. and monitored by PET and SPECT/CT after sequential i.v. injections of 500 μCi [123I]MIBG and 200 μCi [124I]MIBG on days 1 (columns 1 and 2), 8 (columns 3 and 4), and 28 (columns 7–9) or 500 μCi [123I]MIBG alone on day 21 (columns 5 and 6). A, SPECT imaging: images by CT (C, bottom rows) and SPECT (B, middle rows) were fused (A, top rows) to determine the exact location of the SPECT signals. Coronal (columns 1, 3, 5, and 7) and axial (shoulders (columns 2, 4, 6, 8, and 9) and thighs (column 8)) projections are presented. A pinhole collimator was used to acquire SPECT images on day 1. LEHR parallel hole collimators were used thereafter with comparable resolution and increased sensitivity. B, PET imaging: coronal PET images obtained on days 1, 8, and 28 are presented. Both SPECT and PET images show increasing uptake of label from day 1 to day 28 in the HLA-A0201+ tumor (T1). A new migration of CTL to the HLA-DRB,0402+ tumor (T4) is first observed on day 28 by both PET and SPECT. C, accumulation of the radioactivity in the tissue samples obtained from the animals on day 28 after CTL injection confirmed the results of radiotracer accumulation by image analysis, showing CTL-hNETiGFP–mediated [123I]MIBG accumulation in T1 and T4. Significant levels of [123I]MIBG were also detected in the heart and the adrenal gland but not in the spleen (P = 0.05). The table contains P values for paired comparison of statistically significant difference between radiotracer accumulation in the samples of tumor tissues (T1, class I– and class II–matched tumor; T4, class II–matched tumor) versus that in the body tissues or organs designated.
levels of radioactivity were also detected in the T1 tumor, caused by coregistration of the downscatter of the 511-keV photons, produced by $^{[124}\text{I}]$FIAU positron decay, into the SPECT imaging energy window used to measure uptake of $^{123}\text{I}$.

To avoid this downscatter and to differentially image the two types of T cells, mice bearing xenografts of the same two EBV-BLCLs were coinfused with hNET/GFP +CD4+ and HSV-TK/GFP+CD8+ T cells derived from the same EBV-specific T-cell line. Thereafter, infusions of $^{[123}\text{I}]$MIBG and $^{[124}\text{I}]$FIAU and associated SPECT and PET imaging were performed sequentially. Initially, the mice received $^{[123]}\text{MIBG}$ and were imaged by SPECT 4 h later (Fig. 6B). This image detected selective accumulation of $^{[123]}\text{MIBG}$ in the EBV+HLA-A0201+ T2 tumor expected to be targeted by the hNET+CD4+ T cells. Thereafter, each mouse was given 200 μCi $^{[124]}\text{I}]$FIAU and imaged by PET 2 h later. In these PET images, radiolabel was selectively detected in the EBV+HLA-A0201+ T1 tumor (T1) that is targeted by the HLA-A0201–restricted CD8+ EBV-specific T cells (Fig. 6B). Selective accumulation of CD8+ or CD4+ T cells in T1 and T2 tumors, respectively, was confirmed by FACS analysis of T cells isolated from each tumor (Fig. 6C). Thus, this sequential imaging approach permitted clear differentiation of the selective accumulations of two separate T-cell populations transduced to express distinguishable reporter proteins in the same mouse within 6 h on the same day.

**Discussion**

Currently, several reporter gene systems are being used in preclinical models to transduce normal or malignant cells so as to be able to image their migration, growth, and function in vivo (14, 17, 18). However, the clinical applicability of many of these reporters is limited because the proteins they encode are
in vivo

To address this limitation, attention has been focused on identification of human reporter genes encoding enzymes, receptors, or transport proteins with restricted expression in normal tissues, which can mediate differential uptake of radio-labeled drugs or biologicals. Three such genes have been reported previously: (a) hDDR2, by which cells differentially accumulate 3-[(18)F]-fluoroethoxy)-spiperone (3, 6); (b) hSSTR2, expressed by carcinoid tumors, which differentially binds the somatostatin analogue [111In]diethylenetriaminepentaacetic acid-octreotide (4); and (c) hNIS, which mediates differential cellular uptake of either radiolabeled iodide or pertechnetate (2, 5).

In this report, we evaluated the in vivo imaging of adoptively transferred lymphocytes transduced to express hNIS, a human protein also unlikely to be immunogenic in man. The hNIS protein is normally expressed on the plasma membrane of noradrenergic neurons at their synapses and acts to transport norepinephrine by endocytosis from the synaptic cleft back into the neuron. Although these transporters are normally expressed throughout the sympathetic system, cells expressing hNIS are particularly concentrated in the adrenal glands and the heart (12).

In 1967, Morales et al. (21) first showed that [14C]norepinephrine, infused into dogs, selectively accumulated in the adrenal medulla. Subsequently, radiolabeled MIBG, a structural analogue of norepinephrine that can block its neurotransmitter activity, was also shown to accumulate in the adrenal gland and could be used to image this organ (11). In initial clinical trials, it was shown that [123I]MIBG could be used to detect pheochromocytomas (9, 10). It is now also extensively used for imaging and treatment of neuroblastoma (22). In these tumors, uptake of [123I]MIBG is based on their high level of expression of functional norepinephrine transporters.

For our study, we constructed a bicistronic retrovector capable of directing the constitutive expression of hNET and GFP and transduced it into EBV-specific T cells of defined antigen specificity and HLA restriction. Following transduction, T cells expressing the vector-encoded GFP could be isolated in high purity by FACS. In vitro studies showed highly reproducible kinetics of radiotracer uptake in transduced T cells. Exposure of hNET-transduced EBV-specific T cells to MIBG or to MIBG labeled with either 125I or 123I did not alter their cytotoxic activity, confirming our previously published data that showed that the exposure of HSV-TK-transduced EBV-specific T cells to 125I- or 123I-labeled FIAU did not alter their EBV-specific HLA-restricted cytotoxicity in vitro or tumoricidal effect in vivo (14).

In prior studies using this model, we were able to image EBV-specific HSV-TK/GFP–transduced T cells accumulating in targeted tumors by i.v. infusions of [18F]FIAU followed by PET (14). To gain initial estimation of the relative capacity of PET to detect transduced T cells, defined doses of EBV-specific CTL-hNETiGFP and CTL-TKGFP were directly injected into EBV-BLCL tumor xenografts and then imaged after i.v. infusion of [18F]FIAU or [125I]MIBG. Signal intensities detected by PET and accumulations of [125I]MIBG or [123I]FIAU, counted in the tumor sites, were closely correlated with the number of CTL-hNETiGFP or CTL-TKGFP T cells injected (Fig. 2). Furthermore, doses of as few as 10^4 CTL-hNETiGFP or CTL-TKGFP could be clearly imaged. When mice were given [125I]MIBG i.v. and imaged by X-SPECT, signal intensity in the tumors was also closely correlated with dose of T cells down to the lowest dose tested, 10^3 T cells per tumor (Fig. 3). Fusion of CT and SPECT images permitted improved precision in the localization of the radiotracer accumulation sites.

Our group first showed the potential of PET imaging for long-term monitoring of antigen-specific T cells in vivo in studies of the migration of human EBV-specific T cells transduced to express an HSV-TK/GFP reporter gene (14). When transplanted i.v. into NOD/SCID mice bearing EBV+ lymphomas and EBV+ leukemia xenografts of specific HLA genotypes, EBV-specific HSV-TK/GFP T cells selectively accumulated in and induced regressions of EBV+ tumors coexpressing the restricting HLA allele. Sequential PET images obtained after weekly i.v. injections of [124I]FIAU recorded these targeted accumulations of T cells over a period of 15 days after T-cell infusion.

The current study confirms these findings, showing, both by PET and by SPECT imaging, progressive accumulations of hNET+ EBV-specific T cells in targeted EBV+ tumors over a period of 28 days. The present study also provides evidence suggesting that image intensities recorded in vivo by PET and SPECT can be used to estimate the number of T cells accumulating in targeted tumors over time. This is shown by the significant correlations between the number of T cells actually accumulated in the targeted tumor, as measured by quantitation of GFP+ T cells and measurements of radiolabel accumulation in tumors isolated at autopsy, and the number of T cells in the tumor calculated based on the signal intensities measured in PET images of graded doses of T cells directly injected into targeted tumors in vivo. These results are consistent with the studies of Su et al. (23) who measured uptake of [18F]FHBG in mice injected intratumorally with nonspecifically activated murine T cells transduced to express HSV-TK. Based on both measured and calculated numbers of T cells accumulated, T cells increased in the targeted tumor by over 12-fold over the 28 days of observation. In the EBV-specific T-cell line used for this experiment, 14% of the T cells were found to secrete IFN-γ in response to autologous EBV-BLCL. If this represents the true proportion of functional EBV-specific T cells in the line, it can be estimated that 30% of the EBV-specific T cells infused i.v. accumulated in the targeted tumor by day 1 after infusion. The 12-fold increase in T cells detected by day 28 after infusion supports the hypothesis that this progressive accumulation is based largely on proliferation of these T cells at the tumor site.

Recently, other studies in murine tumor models have evaluated the short-term migratory patterns of unsensitized and antigen-specific T cells in vivo using PET to image T cells transduced to express HSV-TK (17, 24) or bioluminescence to track T cells transduced to express luciferase (25, 26). In these studies, T-cell accumulations in targeted tumors have initially been evaluated and detected 3 to 4 days after adoptive transfer into the tumor-bearing mice and have increased over a subsequent 1 to 2 weeks of observation (24). In studies using PET, quantitation of signal intensity emanating from the targeted tumors and other organs has been closely correlated with levels of radiolabel detected in each tissue. However, only one other study has directly correlated signal intensities over time with quantitation of transduced T cells detected in the tissue (24). In that study, Su et al. used sequential PET imaging over 15 days to follow naive and memory-type ovalbumin-specific CD8+ T cells derived from OT-1 mice and expressing a mutant s39-HSV-TK and GFP, after adoptive transfer into mice bearing transgenic tumors expressing ovalbumin. In that study, memory T cells rapidly accumulated in and eradicated...
antigen-positive tumors grown in RAG−/− mice. Although naive T cells from these Tcr transgenic mice could also eradicate these tumors, their accumulation at tumor sites was delayed and quantitatively less than that of the memory T cells. The concentration of T cells required to induce tumor regression was estimated to be $2 \times 10^6$ to $4 \times 10^6$ CD8$^+$ T cells per gram of tumor tissue. Strikingly, such expansions of OT-1 CD8$^+$ T cells to numbers inducing tumor regression occurred in lymphopenic RAG−/− but not in normal wild-type hosts.

The potential utility of in vivo imaging for distinguishing the biological activities of T-cell populations transferred was also highlighted in experiments presented in Fig. 5, which used a line of hNETiGFP$^+$ EBV-specific T cells containing a predominance of HLA-A0201–restricted, EBV-specific double-positive T cells and a small subpopulation of HLA-DRB$^+$0402–restricted, EBV-specific CD4$^+$ T cells. Prior studies have indicated that CD4$^+$CD8$^+$ double-positive human T cells, when detected in the blood, usually represent CD8$^+$ T cells, which up-regulate expression of CD4 during activation in vivo (26). Recent studies have provided evidence that these CD4$^+$CD8$^+$ T cells are potent effector memory T cells that are HLA class I restricted, prominent in individuals chronically infected with HCV or EBV, and particularly concentrated in sites of chronic infection (27). Our findings are consistent with those characterizations, showing, by both PET and SPECT imaging, rapid accumulation of the CD4$^+$CD8$^+$ T cells in EBV$^+$ xenografts coexpressing the restricting HLA-A0201 allele. In contrast, the late accumulation of T cells in the EBV$^+$ xenograft sharing the class II–restricting allele HLA-DRB$^+$0701 likely reflects the small population of EBV-specific cytotoxic CD4$^+$ CD8$^+$ T cells restricted by this allele detected in the T cells infused.

Because interactions between different types of lymphocytes contribute to the kinetics, intensity, antitumor activity, and persistence of adoptive immune responses, a priority for our studies has been the development of technology permitting contemporaneous imaging of such interactions in vivo. In prior studies, Beilhack et al. (28) used bioluminescence imaging to examine the distribution and proliferation of splenic T cells expressing an in vivo luciferase transgene following transplantation into lethally irradiated H-2 disparate allogeneic hosts. During the course of graft-versus-host disease (GVHD), targeted tissues identified by their accumulations of luciferase$^+$ T cells were evaluated by immunohistochemistry for their content of different CD4$^+$ and CD8$^+$ T cells to distinguish the potential of alloresponsive naive and memory T cells to induce GVHD in vivo. However, this single reporter system did not permit continuous monitoring of each T-cell compartment in vivo. Alternative approaches using vectors encoding different bioluminescent reporters [e.g., firefly, Renilla reniformis, and click beetle luciferases (25)], distinguishable by the substrates used and/or by the spectra of light emitted, have been used to contemporaneously monitor more than one transduced cell population in vitro and have been proposed for contemporaneous imaging of two to three cell populations in vivo. Development of such approaches is highly desirable because imaging by bioluminescence can be performed repeatedly at low cost and the background in tissues not containing cells expressing luciferase reporters is extremely low. However, to date, no studies have been reported in which different bioluminescent reporters have been used to successfully discriminate distinct cell populations in vivo. Furthermore, because the transmission of different light spectra through tissues differs, the accuracy of estimates of cell populations expressing certain luciferases when they accumulate in sites distant from the body surface may be limited.

In this report, we show that two T-cell populations bearing hNET and HSV-TK reporter genes can be contemporaneously imaged in vivo over extended intervals using SPECT to detect differentially accumulated $^{[125]}$I-MIBG and PET to detect $^{[124]}$FI AU. Because PET is more sensitive than SPECT when equivalent doses of radiolabel are used, we intentionally increased the ratio of doses of $^{125}$I to $^{124}$I in favor of $^{125}$I-labeled probes and also increased the acquisition time for SPECT images. This approach permitted achievement of a comparable sensitivity of detection in images of the same CTL-hNETiGFP with $^{[125]}$I-MIBG and $^{[124]}$FI AU (Figs. 2 and 3) or equivalent doses of CTL-hNETiGFP and CTL-TKGFP with $^{[125]}$I-MIBG and $^{[124]}$FI AU, respectively. As a result of this modification, in mice bearing EBV$^+$ xenografts differing in their expression of class I and II HLA alleles, we could demonstrate, by SPECT, that the hNET$^+$ EBV-specific CD4$^+$ T cells retaining $^{[125]}$I-MIBG selectively accumulated in EBV$^+$ tumors coexpressing their restricting HLA-DRB$^+$0701 allele. In the same mice, HSV-TK$^+$ EBV-specific CD8$^+$ T cells, which selectively take up $^{[124]}$FI AU by subsequent PET imaging, were shown to have accumulated in EBV$^+$ tumors expressing HLA-A0201. This sequence of imaging permits clear differentiation and quantitation of the accumulated activity of i.v. given $^{[125]}$I-MIBG and $^{[124]}$FI AU in the hNET-transduced CD4$^+$ and HSV-TK–transduced CD8$^+$ T cells, respectively. Although the possibility of such dual imaging has been raised by several groups interested in in vivo imaging techniques (29, 30), this report is, to our knowledge, the first to show its feasibility. Furthermore, because our studies also indicate that the numbers of cells detectable by PET and SPECT are comparable, this approach may be of particular value for accurately measuring the accumulations of two different effector cell populations within a living subject. Both reporter systems used could also be applied clinically. However, additional preclinical trials beyond the scope of the current experiments as well as clinical trials will be required to ascertain the relative merits of each reporter and imaging modality for sequential long-term assessments of adoptively transferred cells in immunocompetent or immunoablated subjects.

Recent reports of leukemias developing in transduced cells in children with X-linked SCID late after treatment with autologous hematopoietic progenitor cells transduced with a retroviral vector encoding a normal IL-2 receptor (γ-chain) have raised concerns about the tumorigenic potential of cells transduced with such vectors (31, 32). Although long-term analyses of retrovirally transduced T cells following adoptive transfer into human patients and mice have thus far not detected either a dominance of integration sites or malignant transformation of transduced cells (33–35), such concerns may limit broad use of such methods for imaging T cells until further and more extended safety data are accrued. Alternatively, other viral vectors or more efficient nonviral strategies, which may reduce or minimize risk of transformation, may be used to introduce reporter genes into these cells (36, 37).

In conclusion, our studies indicate that the hNET vector described induces a level of expression of the hNET that permits effective imaging, with either $^{[125]}$I-MIBG and SPECT or $^{[124]}$FI AU and PET of as few as $10^4$ T cells accumulated in a 1 cm$^3$ tumor nodule. In these studies, the sensitivity of detection was comparable with that achieved by in vivo imaging of T cells transduced to express HSV-TK with PET after i.v. infusion of $^{[124]}$FI AU. We also show the feasibility of contemporaneously imaging the in vivo biodistribution and targeted accumulation of two effector cell
populations in the same animal by separately transducing each effector population to express a distinct reporter protein mediating the uptake of a radiolabeled tracer differentially imaged by SPECT and PET. Lastly, the close correlation observed between quantitation of signal intensity by PET and SPECT and the numbers of T cells directly injected into tumors or detected in targeted tumors after accumulation over time indicates the potential of such imaging strategies for quantitatively estimating the effector cell populations required for effective adoptive immunotherapy.

References


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In vivo Imaging and Quantitation of Adoptively Transferred Human Antigen-Specific T Cells Transduced to Express a Human Norepinephrine Transporter Gene

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