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Optimization of the *PiggyBac* Transposon System for the Sustained Genetic Modification of Human T-Lymphocytes

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Abstract

Optimal implementation of adoptive T-cell therapy for cancer will likely require multiple and maintained genetic modifications of the infused T-cells and their progeny so that they home to and recognize tumor cells, overcome tumor immune evasion strategies and remain safe. Retroviral vectors readily transduce T-cells and integrate into the host cell genome, but have a limited capacity for multigene insertion and co-transduction and are prohibitively expensive to produce at clinical grade. Genetic modification of T-cells using transposons as integrating plasmids is an attractive alternative due to the increased simplicity and cost of production. Of available transposons, *piggyBac* has the higher transposase activity and larger cargo capacity, and we now evaluate *piggyBac* for potential adoptive therapies with primary T cells. *PiggyBac* transposons mediated stable gene expression in ~20% of primary T-cells without selection. Treatment and maintenance of T-cells with IL-15 increased stable transgene expression up to ~40% and expression was sustained through multiple logs of expansion for over 9 weeks in culture. We demonstrate simultaneous integration of two independent transposons in 20% of T-cells, a frequency that could be increased to over 85% by selection of a transgenic surface marker (truncated CD19). *PiggyBac* could also deliver of transposons of up to 13 kb with 10,000 fold expansion of transduced T-cells in culture and finally we demonstrate delivery of a functional suicide gene (iCasp9). *PiggyBac* transposons may thus be used to express the multiple integrated transgenes that will likely be necessary for the broader success of T-cell therapy.

Keywords

Gene transfer; transposons; T cell immunotherapy; genetically-modified T-cells

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INTRODUCTION

Immunotherapy with antigen-specific T-cells can control and eliminate virus infections and Epstein-Barr virus (EBV)-associated lymphoma after hematopoietic stem cell transplantation^{1–4} and has shown promise for the treatment of lymphoma, melanoma and carcinoma arising in the immunocompetent host.^{5–8} Nonetheless, the full potential of viral and tumor-specific T-cells will only be reached when they can withstand the multiple immune evasion mechanisms that viruses and malignant cells have developed to inhibit the function and diminish the survival of these cells.⁹ These active and passive mechanisms include; lack of migration to tumor sites, the secretion of T-cell inhibitory molecules such as transforming growth factor (TGF)- β and indoleamine 2,3-dioxygenase (IDO) by tumors and tumor stroma,^{10–13} and poor expression of antigen and costimulatory molecules required for T-cell stimulation. Genetic modification of T-cells may allow these evasion mechanisms to be counteracted, and expression of transgenic chemokine receptors,¹⁴ dominant negative receptors for inhibitory molecules, shRNAs to inhibit negative signaling molecules and cytokines to maintain T-cell proliferation^{15;16} may all be of value. Similarly, tumors that downregulate MHC molecules and do not present T-cell epitopes, may be targeted by T-cells expressing artificial chimeric antigen receptors (CARs).¹⁷ Since extensive T cell division is an essential part of most successful immune responses, these genetic modifications must be stable and will require vector integration. Hence, there is a concern that such integration will result in genotoxicity due to insertional mutagenesis, particularly if there is a need to express multiple transgenes in a single T-cell as countermeasures to the multiplicity of immune evasion strategies that are displayed by most malignant target cells.

Although T-cells are readily modified by retrovirus and lentivirus vectors, both have limited transgene capacity that allows expression of two or rarely three transgenes.^{18–20} Sequential transduction with several vectors is possible, but the efficiency of co-transduction is lower, the risk of genotoxicity higher, and the manufacturing and testing expenses prohibitive. In addition, expression of transgenes from retroviral vectors in T-cells is dependent on the activation state of the T-cell, and downregulation swiftly follows in the absence of T-cell activation.^{21;22} Plasmid based transposons are an alternative means for T-cell modification. The *Sleeping Beauty* (SB) transposon has been used to express transgenes in T-cells and a clinical trial using T-cells expressing a transgenic CD19-CAR derived from an SB transposon has been approved.^{23;24} Although the costs of manufacturing and testing the encoding plasmids are far lower than for viral vectors, transduction efficiency of SB is such that extensive *ex vivo* selection and expansion is required. By contrast, the *piggyBac* transposon system has been shown in several human and mammalian cells to have greater gene transfer efficiency and also a greater coding capacity than SB.^{25;26;26–31} We now show that *piggyBac* can stably transduce human T-lymphocytes with multiple genes, and that transduced cells can readily be expanded. We also show that a suicide gene can be co-transfected and expressed in these T-cells, further increasing the safety of our approach.

MATERIALS AND METHODS

Plasmid construction

The *piggyBac* (PB)-transposase plasmid, pCMV-PB has been described previously.²⁸ To create PB-transposon plasmids, the inverted terminal repeat (IR) elements of PB were cloned into pIRES2-eGFP (Clontech, Mountain View, CA), which is transcriptionally regulated by the cytomegalovirus immediate early gene enhancer/promoter sequence (CMV).²⁸ Figure 1 shows the transposon plasmids used: pIR-eGFP is a PB-transposon plasmid encoding the internal ribosome entry site (IRES) followed by the enhanced green fluorescence protein (eGFP) and a puromycin resistance cassette. pIR- Δ CD19 is a *piggyBac* -transposon vector encoding IRES and the truncated CD19 (Δ CD19). pIRII- Δ CD19 is identical to pIR- Δ CD19

except that the puromycin resistance gene was deleted. The suicide gene, inducible *caspase-9* (iCasp9) in which human *caspase 9* deleted for its endogenous dimerizing (CARD) domain was fused to a single FK-binding domain has previously been reported.^{32;33} Death of iCasp9-expressing cells can be induced with a small chemical inducer of dimerization (CID) (AP20187; ARIAD Pharmaceuticals, Cambridge, MA, kindly provided by Dr Spencer, Baylor College of Medicine)^{32;33} iCasp9 and Δ CD19 were linked using a 2A-like protease recognition sequence derived from foot-and-mouth disease virus^{34;35}, and were cloned into a *piggyBac*-transposon plasmid (pIRII-iCasp9.2A. Δ CD19). pIR-mTor^{HA}-eGFP encodes the DNA for mTor with a hemagglutinin (HA) tag followed by an IRES element, eGFP and the puromycin resistance gene cassette. All plasmid constructs were confirmed by restriction digestion and DNA sequencing. The *piggyBac* transposase was introduced transiently into T-cells from a separate plasmid to ensure subsequent stability of integrated transposons.

Cell Donors, cell lines and cultures

Peripheral blood mononuclear cells (PBMCs) from healthy volunteers were obtained with informed consent from the Baylor College of Medicine Institutional Review Board. PBMCs were used to generate T-cell blasts or as feeder cells. They were cultured in T-cell media [Advanced RPMI (Gibco-BRL, Gaithersburg, MD) supplemented with 2 mM L-glutamine (GlutaMAX-I, Invitrogen, Carlsbad, CA) and 5% human AB serum]. Artificial K562 cells (aK562), engineered to express CD80, CD86 and 4-1BBL,³⁶ were maintained in RPMI 1640 (Gibco-BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 2mM L-glutamine (GlutaMAX-I, Invitrogen, Carlsbad, CA). aK562 were used as feeder cells to expand *piggyBac*-transduced T-cells in GP40 culture flasks (Wilson-Wolf Manufacturing Inc., New Brighton, MN). Cytokines were added to the cultures where indicated: IL-2 at 50 units per mL, IL-4 at 1000 units per mL, and IL-7 and IL-15 at 10 ng/mL.

Flow cytometric analysis

We stained the transfected or non-transfected T-cells with phycoerythrin (PE)-, peridinin chlorophyll protein (PerCP)-, or allophycocyanin (APC)-conjugated CD3, CD4, CD8, CD16, CD19, CD28, CD45RO, and CD62L monoclonal antibodies (MAbs). All MAbs were purchased from Becton Dickinson (Franklin Lakes, NJ). Control samples labeled with an appropriate isotype-matched antibody were included in each experiment. Cells were analyzed by a FACSCalibur equipped with the filter set for 4 fluorescence signals using Cell Quest software (Becton Dickinson).

Gene transfer into human T-cells

Five million PBMCs were mixed with *piggyBac*-transposon plasmids and/or *piggyBac*-transposase plasmids in the cuvette provided with the kit and then transfected using the Miltenyi Nucleofactor Device (program U-014) in combination with the Human T-cell Nucleofactor Kit according to the manufacture's instruction (Amaxa, Gaithersburg, MD). The amount of transposon and transposase DNA is shown for each experiment. In some experiments, PBMCs were maintained in cytokines overnight before transfection as described. After transfection, PBMCs were stimulated on non-tissue culture-treated 24 well plates coated with 1 μ g/ml OKT3 (Ortho Biotech, Bridgewater, NJ) and 1 μ g/ml anti-CD28 (Becton Dickinson) antibodies (CD3/28 MAbs) in the presence of recombinant human interleukin (IL)-2 (50 IU/mL) (Proleukin; Chiron, Emeryville, CA). After 4 days of stimulation, activated T-cells were transferred to tissue-culture treated 24-well plates in T-cell media supplemented with IL-2 (50 IU/mL) or other growth factors as indicated. Cells were restimulated weekly on CD3/28 MAbs-coated plates. For optimized transduction, PBMCs were rested overnight in 10 ng per mL IL-15 then transfected with 5 μ g of transposase and 5 μ g of transposon DNA, then returned to IL-15 containing medium for 24 hours before activation. On day 1 after nucleofection, transgene-

expressing T-cells were considered “*transfected*”. By day 8, it was assumed that all transgene expressing T-cells had retained integrated *piggyBac* transposon and so at this point, they were considered “*transduced*”. Transfected/transduced cells were harvested for counting and FACS analysis as indicated.

Cell selection and expansion

On day 8 after co-transfection of pIRII-eGFP and pIRII-ΔCD19, T-cells were harvested and incubated with CD19-microbeads (Miltenyi Biotec, Bisley, UK) for 15 minutes at 4 °C, then positively selected using Miltenyi Mini-MACS column according to the manufacture's instructions. The selected cells were immediately re-plated in T-cell media with IL-15 on a CD3/CD28 MAbs-coated 24-well plate and cultured for 2 weeks. On day 22 after transfection, transgene expression and cell subset and memory phenotype were determined. For further expansion, 100,000 T-cells were stimulated with CD3/28 MAbs with aK562 or autologous PBMCs feeder cells in GP40 flasks every 2 weeks. IL-15 (10 ng/mL) was added to the media twice weekly.

Statistical analysis

All data are presented as mean $\pm$ 1 SD. The Student's *t* test was used to determine the statistical significance of differences between samples, and *P* values less than .05 were accepted as indicating a significant difference.

RESULTS

Optimization of *piggyBac* transduction of primary T-cells

We first evaluated the efficiency and toxicity of introducing the *piggyBac* transposon and transposase into primary T cells using nucleofection. We found that both the frequency of gene expression and cell viability were greater after transfection of PBMCs than after transfection of CD3/28 MAbs-activated T-cells (not shown). Hence, in all the following experiments, PBMCs were first transfected and then the T cells they contained were activated with plate bound CD3/28 MAbs. To determine the optimal ratio of *piggyBac*-transposase to transposon DNA, 5×10^6 fresh PBMCs were transfected with 5 μ g of *piggyBac*-transposon plasmid, pIRII-eGFP, and increasing quantities (0, 2.5, 5, or 10 μ g) of *piggyBac*-transposase plasmid, pCMV-*PB*, or 5 μ g of pCMV-*PB* together with increasing quantities (0, 2.5, 5, or 10 μ g) of transposon (pIRII-eGFP). Twenty-four hours after transfection, the cells were counted and activated with CD3/28 MAbs for 7 days. GFP expression was analyzed on day 1 and weekly thereafter. There was significant loss of viability after nucleofection alone; 40% of T cells were dead on day 1 after nucleofection without DNA (not shown). Cell death was increased to about 60% by DNA addition (Figures 2C and 2D). Figures 2A and 2B show that increasing the quantity of both transposon and transposase plasmids increased the frequency of GFP expression at all timepoints, but illustrates that the toxicity also increased with the amount of added DNA (Figures 2C and 2D). The optimal result, a transfection efficiency of 40% to 55% ($47.6 \pm 7.4\%$) and viability of ~40% on day 1, was obtained using 5 μ g each of transposon and transposase, and these quantities were used in future experiments. By day 8 the frequency of transduced cells had declined by about 50%, but thereafter remained stable for as long as the T-cells were cultured (up to 4 weeks of culture shown in Figures 2E and 2F). The reproducible loss of transgene expression from day 1 to day 8 likely represents transfected cells in which the transposon did not integrate and was therefore lost due to cell division by day 8. Certainly, after day 8 it is possible to use plasmid rescue to confirm integration of *piggyBac* into the T-cell genome (See Table, Supplemental Digital Content 1, <http://links.lww.com/JIT/A7>). Since the presence of transposase increased both the frequency and intensity of GFP expression on day 1 (Figures 2A and 2E) the transposase may act as a transactivator of transgene expression.²⁸ While transduced T-cells could be subsequently be maintained in culture over 4 weeks with

stable gene expression (Figures 2A, 2B, 2E and 2F), there was little overall expansion in transduced T-cell numbers (Figures 2E and 2F).

IL-15 increases the viability of transduced T-cells

Since the above studies showed that the toxicity of DNA transfection diminished the initial survival and subsequent expansion of transgene-expressing T cells, we sought to improve the viability of transfected cells. We first determined if incubation of the PBMCs in media containing anti-apoptotic cytokines³⁷ improved cell survival during transfection. We added IL-4, IL-7 and IL-15 to PBMCs both prior to and after transfection, and Figure 3 shows that IL-7 and IL-15 increased both the frequency and number of GFP-expressing cells on day 1 and at all time points thereafter (Figures 3E). Although we still observed a 60% initial loss in viability due to the transfection and a 50% reduction in transgene expression between days 1 and 8, both the frequency of GFP+ T-cells and their subsequent expansion was increased by the addition of cytokines. In three experiments, the increase in the frequency of transduced T cells was 1.27 ± 0.07 fold for IL-7 and 1.43 ± 0.16 fold for IL-15 on day 1, and 1.24 ± 0.28 fold for IL-7 and 1.41 ± 0.28 fold for IL-15 on day 8. Similarly, the increase in transduced cell numbers was 1.55 ± 0.16 fold for IL-7 and 2.01 ± 0.62 fold for IL-15 on day 8. Hence the major benefit of these cytokines was to increase the subsequent expansion of transduced T cells. Overall there were a 2.83 fold higher number of viable transduced cells on day 8 in the presence of IL-15 than in control cultures lacking this cytokine. Since results with IL-15 were consistently although not significantly slightly better than for IL-7 and since the IL-7 receptor, but not the IL-15 receptor is downregulated with time after T cell activation, IL-15 alone was used in all subsequent experiments at all time points. Indeed, when culture was continued in IL-15, the superiority over IL-2 became more apparent, with stable expression of GFP in 40% of T-cells and up to 30-fold expansion of transduced T-cells by day 29 of culture (Figures 3D and 3E). Hence, we used IL-15 in all subsequent experiments.

Increasing transgene numbers

When PBMCs from four different donors were treated with IL-15 and co-transfected with the two separate transposons pIRII-eGFP (a 3.6 kb transposon in a 5.6 kb plasmid and pIRII- Δ CD19 (a 3.9 kb transposon in a 5.4 kb plasmid) together with the transposase plasmid pCMV-*PB* (in a 5.4 kb plasmid) the frequency of cells co-expressing GFP and Δ CD19 on day 1 ranged from 30.4% to 66.6% (mean 46.4%), while an additional 5.5% to 13.2% (mean 10.2%) expressed only GFP and 1.4% to 5.8% (mean 4.8%) expressed only Δ CD19. By day 8, these frequencies had decreased, so that from 5.6% to 22% (mean 14.4%) of T-cells expressed both plasmids, with means of 8.6% and 3.7% of T-cells singly expressing GFP and Δ CD19 respectively. Figure 4A shows a representative experiment in which the level of transduction stabilized between 20–28% from days 8 to 22. Co-transfection with these two small transposons did not significantly decrease cell viability over that obtained with a single small transposon ($49 \pm 8\%$ vs $42 \pm 2\%$). Therefore, *piggyBac* can stably transfer genes from separate transposon plasmids for multigene delivery.

Selection and expansion of transduced T-cells

To obtain large numbers of transduced cells with a high frequency of transgene expression, we co-transfected IL-15-cultured PBMCs with two transposons expressing GFP and Δ CD19 respectively. On day 8, transduced cells were selected based on their expression of CD19 using magnetic beads conjugated to CD19 antibodies, then restimulated on CD3/28 MAbs-coated plates in the presence of IL-15. On day 22, over 80% of cells expressed both transgenes, with the remaining cells expressing only Δ CD19 (Figure 4B). Phenotypic analysis demonstrated variable but apparently non-substantive differences in T-cell subsets and memory markers between cells that were selected and expanded versus cells that were expanded without

selection (a mean of $43 \pm 4\%$ vs $18 \pm 16\%$ of CD4⁺ T-cells, a mean of $61 \pm 7\%$ vs $86 \pm 11\%$ of CD8⁺ T-cells, a mean of $55 \pm 10\%$ vs $35 \pm 8\%$ of CD28⁺ T-cells and a mean of $34 \pm 14\%$ vs $34 \pm 12\%$ of CD62L⁺ T-cells) (Figures 4C and D). However, for all donors, both CD4⁺ and CD8⁺ and memory T-cells were represented in transgene-positive populations.

Enhanced expansion of *piggyBac*-transduced T-cells using feeder cells

Although *piggyBac*-transduced T-cells could be expanded by culture in IL-15, their rate of expansion remained lower than for non-transfected T-cells. For many T-cell therapy applications, large T cell numbers are required, and over 10^{10} T-cells have been given to human subjects in several clinical trials.^{1,38–40} To rapidly expand transgene-expressing T-cells, we compared the efficacy of two different irradiated feeder cell types; autologous PBMCs and aK562 cells modified to express the costimulatory molecules CD80, CD86, CD83 and 4-1BB ligand.³⁶ Fifteen million PBMCs cultured overnight with IL-15 were co-transfected with pIRII-eGFP, pIRII-ΔCD19, and pCMV-PB. 6.0 to 6.2×10^6 (mean 6.3×10^6) transfected cells were recovered on day 1 and by day eight, 17.1 to 27.0×10^6 (mean 21.9×10^6) cells were recovered, of which 6% to 17% remained GFP⁺ CD19⁺ double-positive. ΔCD19-expressing cells were selected on day 8 using anti-CD19-microbeads and 4.3×10^5 cells were recovered. 1×10^5 cells were activated with CD3/28 MAbs and co-cultured with either 30 Gy irradiated autologous PBMCs or with 80-Gy irradiated aK562 at a responder: feeder ratio of 1:20. Cultures were fed twice weekly with IL-15. Two weeks later, cells were counted and analyzed for GFP and ΔCD19 expression. Similar frequencies of GFP-expressing T-cells were obtained by culture on PBMCs and aK562 feeders (Figure 5A), while a range of 165 to 422 fold expansion (mean 296 fold) of GFP+CD19⁺ transduced T-cells was achieved after culture on aK562 cells, compared with 16 to 400 fold expansion (mean 155) on PBMCs feeders. After restimulation with CD3/28 MAbs every two weeks, cells could be further expanded on aK562 feeder cells at a similar rate for up to 50 days to produce an extrapolated expansion of up to 8 logs (calculated from small aliquots of T cells maintained in culture) (see Figure 5B) with over 80% of T-cells expressing both transgenes by day 64 (see Figure 5C).

T-cells can be transduced with large transposons and expanded

Expression of a large single or multiple small transgenes in T-cells could be achieved using a single large transposon. To determine how the size of the transposon plasmid affected transfection efficiency and cell expansion, we tested GFP expression from increasingly large transposons. We found that increasing transposon size from 5.2 kb in a 7.2 kb plasmid (pIR-eGFP) to 13 kb in a 15 kb plasmid (pIR-mTor^{HA}-eGFP) consistently decreased the percentage of GFP⁺ cells at day 1 (range 32% to 47% and 15% to 37% respectively). By day 8, the percentage of transduced cells was similar between the two vectors (range 5% to 16% for both vectors, see Figure 6A), but the expansion of cells transfected with the larger vector consistently declined over the first 8 days of culture (Figure 6B), likely reflecting the toxicity of the large plasmid. However, after sorting for GFP expression on day 8, all transfected cells expanded similarly and rapidly (up to 3 logs in 14 days) and maintained GFP expression (85% for pIR-eGFP and 81% for pIR-mTor^{HA}-eGFP on day 14 after sorting) (Figure 6C). They expressed transgenic mTor protein as shown by western blotting in Figure 6D, indicating that large transposons can be integrated into T cells and do not hinder T-cell expansion once they are stably integrated.

Selection of T-cells expressing a functional suicide gene

Since any transduced T cell has the potential for long term survival and expansion in vivo, and hence for sustained and progressive adverse effects, we evaluated the function of the inducible *caspase 9*, (iCasp9) suicide gene when it was inserted in the *piggyBac* transposon together with a selectable marker, ΔCD19.^{32;41} IL-15-treated PBMCs were co-transfected with three

transgenes on two transposons, pIRII-iCasp9.2A. Δ CD19 (5 μ g), pIRII-eGFP (5 μ g), and pCMV-*PB* (5 μ g). Non-transfected PBMCs were used as controls. After 7 days stimulation with CD3/28 MAbs in the presence of IL-15, the CID was added at 50 nM. After one week, we analyzed the cells for their expression of Δ CD19, which was expressed from the same transposon as iCasp9. Cells expressing eGFP alone from the second transposon were unaffected by the dimerizer drug and survived in the culture, but the frequency of CD19+ T-cells in the population that received pIRII-iCasp9.2A. Δ CD19 was reduced from 20% to less than 1% (Figure 7). This was below the percentage of CD19+ cells in the control eGFP alone-transduced population and likely represented contamination from normal CD19+ B cells rather than a surviving transduced population.

DISCUSSION

Our results show effective non-viral genetic modification of primary human T lymphocytes using the *piggyBac* transposon system. Stable reporter gene expression was initially around 20%, but after culture optimization and addition of IL-15, this level increased to ~40%, even without selection. We showed efficient multigene delivery using two separate transposons and stable co-expression of two separate genes in up to 28% of T-cells. After magnetic bead selection, we obtained stable expression of genes from two separate transposons at >80% efficiency in cells which could be rapidly expanded in culture, for over 50 days whilst maintaining stable transgene expression. We also demonstrate the feasibility and functionality of *piggyBac*-mediated delivery of an inducible suicide gene into human T-cells.

The major difficulty we faced was the toxicity of the transduction procedure. Nucleofection itself is toxic to T cells and the addition of DNA further decreased cell survival to about 40% by 24 hours after transfection. More importantly it also reduced the subsequent expansion rate of both transduced and non-transduced T cells. Increasing the amount of DNA and the size of the transposon plasmid further increased this toxicity. Other methods of gene transfer produce significantly less toxicity than nucleofection. Lentivirus and retrovirus vectors have high transduction efficiency in human T cells and low toxicity,⁴² and have been used successfully in T-cell gene transfer studies, both for therapeutic transgenes and for the transmission of marker genes.^{43;44} However lentiviruses and retroviruses have a smaller cargo capacity and are prohibitively expensive to manufacture at clinical grade. Lipofection approaches have less toxicity than electroporation, but have poor efficacy with suspension cells and efficient lipofection of primary human T-cells has not been reported.⁴⁵

Given the potential advantages of transposons, we sought means of improving culture conditions, to compensate for the toxicity. Although the addition of survival-promoting cytokines such as IL-15 or IL-7 did not appear to decrease the initial adverse effects of nucleofection, these agents increased both the frequency of gene expression and the ability of the transduced T cells to expand. These cytokines are known to induce the expression of anti-apoptotic molecules like bcl2 that promote T cell survival.^{46;47} T-cell expansion could be improved further by the use of feeder cells and both autologous PBMCs and K562 cells modified to express the costimulatory molecules, CD80, CD86 and 4-1BB ligand proved highly effective.³⁶ Autologous PBMCs are the simplest source of feeder cells, but PBMCs cannot be standardized, large cell numbers may be required, making scalability difficult, and cancer patient PBMCs may contain negative regulatory components. aK562 cells are HLA class I and class II-negative and therefore do not induce alloreactivity or skew the antigen specificity of the T-cell population.⁴⁸ aK562 reproducibly enhanced growth rates to a greater level than PBMCs. This difference is likely because aK562 cells express costimulatory molecules at a higher level than PBMCs, in which these molecules are largely limited to monocytes and B cell subpopulations. Gene-modified K562 cells have already been used as tumor vaccines in clinical trials⁴⁹ and so the use of this cell line for the expansion of clinical grade T-cells is

feasible. Using a combination of *piggyBac* transduction and aK562 feeder cells and a proposed starting blood volume of ~50 mL, initial yields of about 2×10^6 selected, stably transduced T-cells could be expanded by 4 to 5 logs to over 10^{10} transduced T-cells in four to five weeks and to 10^{12} in six to seven weeks. These numbers would be more than adequate for any current T-cell transfer clinical study.^{3;4;43;50}

The *piggyBac* strategy was effective even for large transgenes like mTor (Figure 6). This is in contrast to our previous failed attempts to transfer mTor into human T-cells using lenti- or retrovirus vectors (unpublished observations). We are currently redesigning our transposons to eliminate unnecessary DNA sequences and to include the *piggyBac* enzyme within the plasmid, but outside of the transposon sequences so that the transposase is not integrated. Other modifications of plasmids that may reduce size and toxicity include DNA condensation, the use of minicircles in which the DNA backbone is eliminated and reduction of CpG sequences that may activate toll-like receptors.⁵¹

Transposons dramatically increase the rate of integration of transfected DNA into cells compared to that of standard plasmids. Previous reports have evaluated the *SB* transposon for the gene modification of human T-cells. Huang et al.⁵² described 3–10% stable reporter gene expression in T-cells and subsequently 1–3% expression of chimeric antigen receptors (CARs) in T-cells that could subsequently be selected for expansion by flow cytometry.⁵³ Singh et al also used *SB* to express CD19-CAR, but did not evaluate gene expression until day 28 after CD19 antigen-driven expansion, at which time CD19-CAR was expressed in over 25% of T-cells.²³ Further antigen driven expansion in the presence of IL-15 led to CD19-CAR expression in 99% of T-cells by day 70, with retention of memory markers on subpopulations of T-cells. Our own data show stable transfection of T cells without cell selection and transgene expression remains stable for at least 9 weeks, regardless of the T cell activation state. This is in contrast with the downregulation of transgene expression from retrovirus vectors that occurs in human T cells in the absence of antigenic stimulation.^{21;22}

The capacity to select T-cells carrying suicide genes increases the safety of gene modified T-cells. If transgenes are to be expressed from multiple transposons, the suicide gene must be expressed in all transduced cells. This requires first, that a significant number of cells are transfected with the transposase plasmid as well as with multiple transposon plasmids and second, that the transposase can integrate two or more transposons. Our data confirm that *piggyBac* can stably lead to expression of multiple transposons in each T cell, data which replicate our earlier observations in human embryonic kidney cells,²⁸ and that are also consistent with their recent use to generate inducible pluripotent stem cells from normal skin fibroblasts by transfer of canonical ES cell transcriptional regulators.^{25;28;30}

The potential for *piggyBac* to stably transduce T cells with multiple genes is important for successful cancer therapy. Although adoptively transferred T-cells have regularly proved able to control virus infections after stem cell transplantation and to produce complete remissions in EBV-associated lymphoma and nasopharyngeal carcinoma as well as in melanoma, extension to other cancers has proved more difficult, due to their multiplicity of immune evasion strategies. The ability to introduce multiple countermeasures into the transferred T cells and their progeny may therefore broaden the applicability of adoptive T cell therapies to many additional tumors. Adoption of *piggyBac* transposons for this purpose should be facilitated by their lower costs and high efficiency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Schema of *piggyBac*-transposon and transposase plasmids used to transfect PBMCs. The pIR-eGFP, pIRII-eGFP, pIRII- Δ CD19, pIRII-iCasp9.2A. Δ CD19 and pIR-mTor^{HA}-eGFP are *piggyBac*-transposon plasmids. The pCMV-PB is a *piggyBac*-transposase plasmid. IR, inverted terminal repeat; MCS, multiple cloning site; CMV, cytomegalovirus immediate early promoter; IRES, internal ribosomal entry site; eGFP, enhanced green fluorescent protein; pA, polyadenylation sequence; Δ CD19, truncated CD19; iCasp9, inducible *caspase-9*; 2A, 2A self-processing peptide derived from the foot-and-mouth disease virus; puroR, puromycin resistance gene cassette; HA, hemagglutinin tag.

FIGURE 2.

Optimization of DNA concentrations in *piggyBac* transfection of T-cells. To optimize transposase and transposon DNA concentrations, PBMCs were transfected with 5 μ g of pIRII-eGFP and 0, 2.5, 5, or 10 μ g of pCMV-PB (A, C and E) or 5 μ g of pCMV-PB and 0, 2.5, 5, or 10 μ g of pIRII-eGFP (B, D and F) respectively. Transfected cells were stimulated with CD3 and CD28 MAbs in the presence of IL-2 and harvested for transgene analysis and counting weekly between days 1 to 29 after transfection (A and B). The growth kinetics of total (C and D) and GFP+ (E and F) T cells are shown. Data represent the mean $\pm$ SD of 3 donors.

FIGURE 3.

Improved viability of transduced T-cells using cytokines. Untreated or cytokine-pre-treated PBMCs were transfected with pIRII-eGFP (5 μ g) and pCMV-*PB* (5 μ g), then stimulated with CD3/28 MAbs in the presence of the same cytokine as used for pre-treatment or IL-2 (control). Cells were counted and analyzed for GFP expression by flow cytometry on day 1 and day 8 and weekly thereafter. Effects of cytokines on the frequency (A), and number (B) of transgene expressing cells and total cells (C) on day 1 and day 8 after transfection are shown. Longer term proliferation of the transduced T-cells PBMCs in the presence of IL-15 or IL-2 (Control) is shown in D and E. Data represent the mean $\pm$ SD of 3 donors.

FIGURE 4.

Expression and selection of 2 transgenes from 2 plasmids. PBMCs were cotransfected with pIRII-eGFP (5 μ g), pIRII- Δ CD19 (5 μ g), and pCMV-*PB* (5 μ g). The co-transfected cells were stimulated with CD3/28 MAbs in the presence of IL-15. A, The cells were analyzed for GFP and CD19 expression by flow cytometry on day 1 and weekly thereafter. B, On day 8 a fraction of the T cells were selected for expression of CD19 using magnetic beads, and then immediately stimulated on CD3/28 MAbs-coated plates in the presence of IL-15 for 2 weeks. The cells were analyzed for GFP and CD19 expression on day 22. At this time the T-cell phenotype of selected and unselected cells was compared using the subset markers, CD4 and CD8, and memory markers, CD28 and CD62L, as shown in C and D.

FIGURE 5.

Expansion of selected transgenic T-cells. PBMCs were co-transfected with pIRII-eGFP, pIRII-ΔCD19 and pCMV-PB and then selected for CD19 expression on day 8. The selected cells were immediately transferred into 30 mL of T-cell media with IL-15 in a GP40 flask which had been pre-coated with CD3/28 MAbs together with 30 Gy-irradiated autologous PBMCs or 80-Gy irradiated aK562 at a responder: feeder cell ratio of 1:20 for 2 weeks. A, T-cells were counted and analyzed for GFP and CD19 expression on day 22 after co-transfection. B and C, A fraction of the transduced T-cells were expanded on aK562 cells in GP40 flasks for up to 64 days of total culture. The potential total number of cells on day 50 was extrapolated for part (B). The stability of dual transgene expression over the 64 days in culture with IL-15 and irradiated aK562 feeder cells is shown in C.

FIGURE 6.

PiggyBac can stably transfer large genes. IL-15-treated PBMCs (from 4 healthy donors) were co-transfected with 5.7 μ g pCMV-*PB* and 5 μ g pIR-eGFP (5.7 kb transposon in a 7.2 kb vector), plus 5.6 μ g pUC19 (as filler DNA) or 10.6 μ g pIR-mTor^{HA}-eGFP (13 kb transposon in a 15 kb vector) and stimulated with OKT3 (50 ng/mL) on feeder cells in the presence of IL-15 (10 ng/mL). The percentage of transfected (GFP+) cells (A) and total cell number (B) was determined at 1 and 8 days post-transfection by FACS analysis and counting by trypan blue exclusion, respectively. Transfected cells were enriched in each culture to >80% after sorting for GFP+ cells. Sorted GFP+ cells from the pIR-eGFP and pIR-mTor^{HA}-eGFP transfections expanded similarly and rapidly after stimulation with OKT3 on feeder cells and maintained GFP expression (shown in parentheses above each data point) (C). Mock transfected cells were subjected to the transfection procedure without DNA and were not sorted. Expansion data is representative of 3 healthy donors. D is a western blot showing the expression of mTor^{HA} in transduced T cells using an antibody to the HA tag.

FIGURE 7.

Safety switch: Function of suicide transgene. IL-15-treated PBMCs were co-transfected with a suicide gene-encoding plasmid, pIRII-iCasp9.2A.ΔCD19 (5 μg), pIRII-eGFP (5 μg), and pCMV-PB (5 μg) and then stimulated with CD3/28 MAbs in the presence of IL-15. On day 8 the chemical inducer of dimerization (CID) AP20187 was added to the cultures at a concentration of 50 nM. After one week of treatment with CID, we analyzed for GFP and CD19 expression by flow cytometry on day 15.