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Research Articles

Efficient Clinical Scale Gene Modification via Zinc Finger Nuclease–Targeted Disruption of the HIV Co-receptor CCR5

Dawn A. Maier,[1,*] Andrea L. Brennan,[1,*] Shuguang Jiang,[1,*] Gwendolyn K. Binder-Scholl,[1,†] Gary Lee,[2] Gabriela Plesa,[1] Zhaohui Zheng,[1] Julio Cotte,[1] Carmine Carpenito,[1,‡] Travis Wood,[2] S. Kaye Spratt,[2] Dale Ando,[2] Philip Gregory,[2] Michael C. Holmes,[2] Elena. E. Perez,[1,§] James L. Riley,[3] Richard G. Carroll,[1,¶] Carl H. June,[1] and Bruce L. Levine[1]

Abstract

Since HIV requires CD4 and a co-receptor, most commonly C-C chemokine receptor 5 (CCR5), for cellular entry, targeting CCR5 expression is an attractive approach for therapy of HIV infection. Treatment of $CD4^+$ T cells with zinc-finger protein nucleases (ZFNs) specifically disrupting chemokine receptor CCR5 coding sequences induces resistance to HIV infection *in vitro* and *in vivo*. A chimeric Ad5/F35 adenoviral vector encoding CCR5-ZFNs permitted efficient delivery and transient expression following anti-CD3/anti-CD28 costimulation of T lymphocytes. We present data showing CD3/CD28 costimulation substantially improved transduction efficiency over reported methods for Ad5/F35 transduction of T lymphocytes. Modifications to the laboratory scale process, incorporating clinically compatible reagents and methods, resulted in a robust *ex vivo* manufacturing process capable of generating $>10^{10}$ *CCR5* gene-edited CD4+ T cells from healthy and HIV+ donors. CD4+ T-cell phenotype, cytokine production, and repertoire were comparable between ZFN-modified and control cells. Following consultation with regulatory authorities, we conducted *in vivo* toxicity studies that showed no detectable ZFN-specific toxicity or T-cell transformation. Based on these findings, we initiated a clinical trial testing the safety and feasibility of *CCR5* gene-edited CD4+ T-cell transfer in study subjects with HIV-1 infection.

Introduction

Highly active antiretroviral therapy (HAART) controls HIV replication and generally improves immune status in people who are HIV+, significantly prolonging survival. HAART is a lifelong drug therapy, with difficulties in medication adherence and long-term toxicities. However, many patients still present late, which is associated with diminished immune restoration and shorter survival durations. These patients would benefit from immune restoration in addition to antiretroviral therapy to address the immune activation and incomplete immune restoration that persists during HAART.

Immune-based therapies are attractive since there is evidence that control of HIV-1 infection is associated with strong virus-specific polyfunctional CD4+ T cells that support antiviral CD8+ T cells (Pantaleo and Koup, 2004). We have shown that reconstituting CD4+ helper T-cell activity through adoptive transfer of costimulated CD4+ T cells can improve CD4 counts and may augment natural immunity to HIV-1 infection (Levine *et al.*, 2002). A treatment modality that protects CD4+ T cells from HIV infection *in vivo* may result in improved antiviral immunity as well as overall immune function and reduction in disease-related morbidity.

Gene therapy for HIV-1 infection, including antisense RNA, transdominant proteins, ribozymes, RNA decoys, single chain antibodies, and RNAi (RNA-interference), has long been proposed as an alternative to antiretroviral drug regimens (Sarver and Rossi, 1993; Dropulic and June, 2006). Payloads targeting entry of HIV have also been investigated

*These authors contributed equally to this work.
[1]Department of Pathology and Laboratory Medicine, [3]Department of Microbiology, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA 19104.
[2]Sangamo BioSciences, Richmond, CA 94804.
[†]Current affiliation: Adaptimmune LLC, Philadelphia, PA 19104.
[‡]Current affiliation: Lonza, Walkersville, MD 21793.
[§]Current affiliation: University of Miami Miller School of Medicine, Miami, FL 33136.
[¶]This manuscript is dedicated to the memory of our friend and colleague Richard Carroll.

both in preclinical studies and in human trials (Li *et al.*, 2005; van Lunzen *et al.*, 2007). Blocking HIV at an early step in the replication cycle may be important for conferring a selective advantage to genetically modified cells in the body and hence allow outgrowth of HIV-resistant cells *in vivo* (von Laer *et al.*, 2006).

A naturally occurring mutation of the HIV entry co-receptor C-C chemokine receptor 5 (*CCR5*; Δ32) that explains why certain high exposure-risk patients remained HIV-resistant was described in 1996 (Liu *et al.*, 1996). The homozygous 32-base-pair (bp) deletion in *CCR5* results in a dysfunctional receptor (Quillent *et al.*, 1998; Carrington *et al.*, 1999). CD4 T cells from individual's homozygous for the *CCR5Δ32* mutation required a 100-fold higher level of HIV to be infected (Paxton *et al.*, 1996). In HIV+ long-term non-progressors (defined by the criteria of HIV positivity for >7 years, CD4 cell counts >600, no antiretroviral therapy, and no symptoms), the frequency of *CCR5Δ32* heterozygotes is significantly higher than in the general population, indicative of a protective effect in heterozygotes (Cohen *et al.*, 1997; Eugen-Olsen *et al.*, 1997). The functional eradication of HIV-1 in a 40-year-old HIV-positive man in Berlin who underwent hematopoietic stem and progenitor cell (HSPC) transplantation from a homozygous *CCR5Δ32* (CCR5 – /CCR5 –) donor and achieved sustained virologic suppression without antiretroviral therapy has increased the rationale for immune-based therapies of HIV-1 infection that target CCR5 (Hutter *et al.*, 2009; Hutter and Thiel, 2011; June and Levine, 2012).

Designed zinc-finger protein (ZFP) domains have been successfully used as engineered transcription factors to modulate endogenous gene expression by fusion to well-characterized transcriptional activation or repression domains (Liu *et al.*, 2001; Rebar *et al.*, 2002; Snowden *et al.*, 2003). Importantly, engineered ZFPs can bind with singular specificity (Tan *et al.*, 2003) and fusion of an engineered ZFP to the catalytic domain of the type IIS restriction enzyme FokI creates a designer zinc finger nuclease capable of cleaving DNA specifically at a unique and predetermined site in the human genome (Kim *et al.*, 1996; Smith *et al.*, 1999; Urnov *et al.*, 2005; Rahman *et al.*, 2011). We previously showed that engineered ZFNs targeting human *CCR5* efficiently generate a double strand break at a predetermined site in the *CCR5* coding region upstream of the natural *CCR5Δ32* mutation. Transient expression of the *CCR5*-targeted ZFNs was sufficient to selectively, efficiently, and stably modify the *CCR5* locus in both primary T cells and T-cell lines. In addition, ZFN-modified T cells show a marked growth advantage when challenged both *in vitro* and *in vivo* with CCR5-tropic HIV (Perez *et al.*, 2008).

The studies presented here have moved the technology of disruption of *CCR5* by engineered ZFNs from the research bench to clinical scale using good manufacturing practice (GMP)-compliant reagents, supplies, and procedures. Following CD3/CD28 stimulation and Ad5/F35 adenoviral vector transduction, more than 1×10^{10} *CCR5* gene-edited CD4+ T cells from healthy and HIV-1 infected donors can be generated. CD4+ T cell phenotype, function as assayed by cytokine production and repertoire were comparable between ZFN-modified and control cells. *In vivo* toxicity studies showed no detectable ZFN-specific toxicity or T-cell transformation. Based on these data and following regulatory approval by the National Institutes of Health (NIH) Recombinant DNA Advisory Committee, University of Pennsylvania Institutional Review Board (IRB) and Institutional Biosafety Committee (IBC), and Food and Drug Administration Center for Biologics Evaluation and Research (FDA-CBER), we initiated a Phase I clinical trial testing this first use of ZFNs in HIV-1 infected subjects (www.clinicaltrials.gov NCT00842634).

Material and Methods

Leukapheresis or whole blood collection and cell separation

Leukapheresis was performed on donors consented on institutional IRB-approved protocols using a Baxter CS3000 (Baxter, Deerfield, IL) or a COBE Spectra (CaridianBCT, Lakewood, CO) in the apheresis unit at the Hospital of the University of Pennsylvania. Leukapheresis cell products were elutriated within 24 hr of collection using the Elutra® Cell Separation System (CaridianBCT) using a protocol developed to maximize lymphocyte recovery and purity (Powell Jr. *et al.*, 2009). For the experiments shown in Figure 1 and Table 1, whole blood from HIV-1+ or healthy donors was collected via venipuncture, and peripheral blood mononuclear cells (PBMC) were isolated using Lymphocyte Separation Media (Lonza, Walkersville, MD). PBMC, peripheral blood lymphocytes (PBL), or elutriated lymphocytes next underwent CD8 depletion to enrich for CD4+ T cells. For smaller scale experiments, CD8 T-cell depletion was performed using the Miltenyi MidiMACS® System, LD columns, and research grade CD8 microbeads (Miltenyi, Auburn, CA) according to the manufacturer's instructions. For clinical scale validation and engineering runs, CD8 depletion was performed on the Miltenyi CliniMACS® system following CD8 enrichment 1.1 protocol and the negative fraction retained for *ex vivo* stimulation and transduction as described below. Thirteen unique human HIV-1+ and healthy donors were used for the studies shown in figures and tables (Supplementary Table 2; Supplementary Data available online at www.liebertonline.com/hum); altogether >20 unique donors were used in preclinical process development, validation, and engineering studies.

Media and culture conditions

CD4-enriched lymphocytes were initially seeded into XVIVO™ 15 media formulated without Phenol Red or Gentamicin (Lonza, Walkersville, MD) and supplemented to a final concentration of 20 m*M* HEPES (Lonza), 2 m*M* L-Glutamax (Invitrogen, Carlsbad, CA), 0.5% human serum albumin (CSL Behring, Kanakakee, IL), 10 m*M* N-acetylcysteine (Roxane Laboratories, Columbus, OH), 1 m*M* sodium pyruvate, 1% minimal essential vitamin mix (both from Lonza), 500 n*M* Norvir® (ritonavir; Abbott Laboratories, North Chicago, IL), 1 μM Retrovir® (zidovudine; GlaxoSmithKline, Research Triangle Park, NC), and 100 IU/mL of Interleukin-2 (Chiron Corp, Emeryville, CA). This media formulation also served as Ad5/F35 vector transduction medium. At Day 3 of culture and thereafter, cells were fed with XVIVO™-15 supplemented as above, except that 5% pooled human AB serum (Valley Biomedical, Winchester, VA) was substituted for 0.5% human serum albumin, with 100 IU to 500 IU/mL of

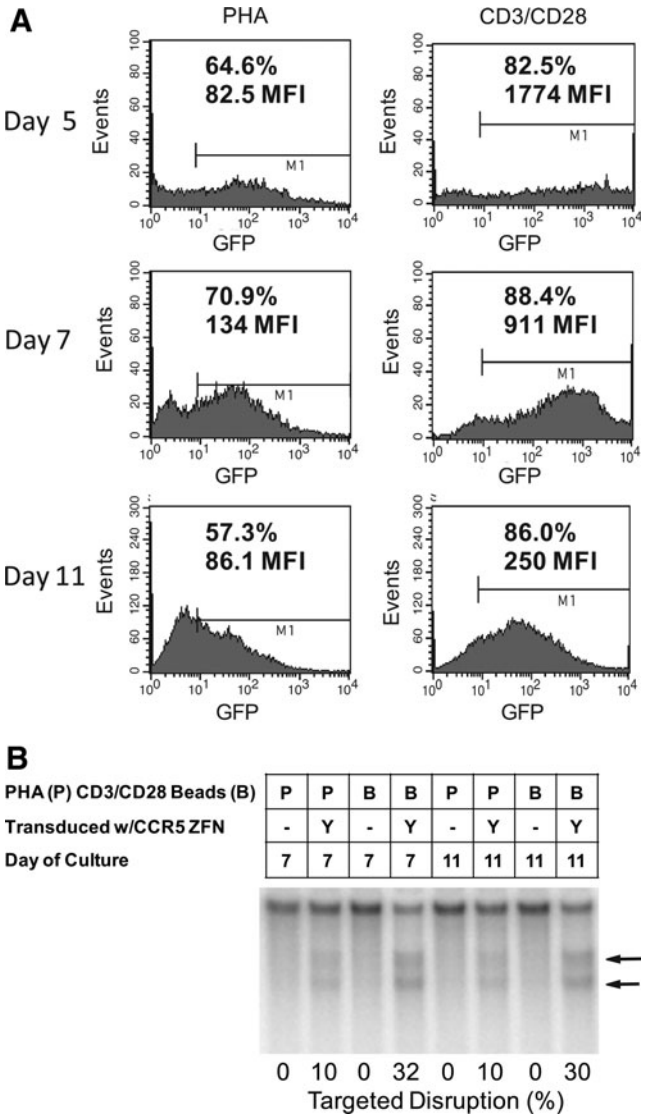

FIG. 1. Effects of the method of T-cell activation on Ad5/F35 vector transduction and expression efficiency. Primary CD4 T cells were activated overnight with 5 μg/ml phytohaemagglutinin (PHA) or anti-CD3/28 antibody-conjugated beads at a 3:1 bead:cell ratio and then infected at a multiplicity of infection (MOI) of 600 with Ad5/F35 vector. **(A)** Ad5/F35 vector-expressing green fluorescent protein (GFP) was used to measure transduction efficiency by fluorescence activity at Days 5 to 11 post-stimulation. The percent of cells expressing GFP are shown, as well as the mean fluorescence intensity (MFI), which reflects the level of GFP expression in each cell. **(B)** Ad5/F35 vector expressing the *CCR5*-specific ZFN, SB-728, was used to measure nuclease activity after transduction. Boxes above the gel show the method of T-cell stimulation, ZFN treatment, and the day of culture. Nuclease activity was measured by using the surveyor nuclease assay on *CCR5*. Cleavage fragments are indicated by the arrows to the right of the gel. Percent disruption was determined by image analysis as described in the methods and is indicated at the bottom of the gel.

Interleukin-2 added, depending on the culture cell concentration. For clinical scale experiments, on Day 5, once cells were seeded into the WAVE™ Bioreactor, 0.02% Pluronic (CellGro®, Manassas, VA) was added to the media. Cell counts and size determinations were performed on a Coulter Multisizer™ 3 (Beckman Coulter Particle Characterization, Miami, FL). Cell viability was assessed by Trypan blue dye exclusion.

Table 1. Zinc Finger Nuclease–Mediated Genome Disruption of *CCR5* at Five Days Following PHA or CD3/CD28 Stimulation (Percent of Alleles Disrupted)

	PHA	*CD3/CD28*
Donor 1 (healthy)	4.1	16.1
Donor 2 (healthy)	7.3	24.6
Donor 3 (HIV-1+)	10.4	37.4

PHA, phytohaemagglutinin; CCR5, C-C chemokine receptor 5.

T-cell activation and adenoviral transduction

CD4-enriched lymphocytes were seeded at 1×10^6 per mL in transduction media and stimulated with anti-CD3 and anti-CD28 mAb-conjugated beads as described (Levine *et al.*, 1997) or phytohaemagglutanin (PHA; Sigma, St. Louis, MO) at 5 ug/mL and incubated in a 37°C, 5% CO_2 incubator. Following overnight incubation, cells were transduced with either Ad5/F35 CCR5-ZFN or Ad5/F35 green flourescent protein (GFP) vector. Cells were cultured in either T25 plastic flasks for research scale or Baxter Lifecell flasks (Baxter, Deerfield, IL) for clinical scale experiments. At culture Day 3 and after, cells were counted and fed with fresh media containing human serum. For clinical-scale experiments, the cells were transferred to the WAVE Bioreactor™ 2/10 (GE Healthcare Bio-Sciences, Piscataway, NJ). Seeding concentration for the WAVE 2/10 was 0.5×10^6/mL, and media perfusion was initiated once the culture had reached maximum bag volume. When the cell culture reached 5×10^6 cells per mL, perfusion was increased to one culture volume per 24 hr. For both research scale and clinical scale experiments, cells were cultured for 9–12 days before harvest. For clinical scale, anti-CD3/anti-CD28 beads were removed with a MaxSep Magnetic Cell Separator (Baxter), and cells were concentrated and washed with a Baxter Fenwal Harvester (Levine *et al.*, 1998) and subsequently cryopreserved using a controlled rate freezer.

Ad5/F35-GFP and Ad5/F35-CCR5-ZFN vectors

The ZFNs targeting the human *CCR5* gene were designed and assembled as previously described (Perez *et al.*, 2008). The CCR5-ZFNs were inserted into the pAdEasy-1/F35 vector linked together via a 2A peptide sequence and under control of the tet-regulated cytomegalovirus internal promoter (Invitrogen) to regulate transgene expression during vector production. The *GFP* reporter gene was independently inserted into the pAdEasy-1/F35 vector to create the Ad5/F35-GFP vector. The Ad5/F35 vectors were packaged in TREx 293 cells (Invitrogen), essentially as previously described (Nilsson *et al.*, 2004).

Flow cytometry

Cells for analysis were resuspended in flow cytometry staining medium (3% heat-inactivated pooled human AB serum and 0.05% sodium azide along with 2m*M* EDTA in PBS w/o Ca or Mg) prior to the addition of staining antibody. Cell samples were incubated in the dark for 30 min at

4°C, washed with staining buffer, and resuspended in either staining buffer or 1% paraformaldehyde. Cell acquisition was performed using a Becton Dickinson Biosciences (San Jose, CA) FACSCalibur flow cytometer and accompanying BD CELLQuest Pro software. Antibodies were purchased from BD Biosciences. T cells were defined as CD3+ gated on CD45+ cells.

Cytokine analysis

Following 9–12 days of culture, 2×10^6 cells from either transduced ZFN-modified or nontransduced control cultures were washed, resuspended in fresh media, and either stimulated with fresh anti-CD3/anti-CD28 mAb beads or left unstimulated (control), each at a final cell concentration of 1×10^6 per mL. Supernatants were harvested 24 hr later. Cytometric bead array reagents in Th1/Th2 kits or Flex Sets designed for multiplex analysis (Becton Dickinson) were used to measure concentrations of IL-2, TNF-α, Interferon-γ, MIP-1α, MIP-1β, GM-CSF, and RANTES. Approximately 300 beads per cytokine were collected and analyzed using the FCAP Array™ software.

Surveyor nuclease assay

The Surveyor nuclease assay to detect genetic modification of sites in the genome by the CCR5-ZFNs was performed as previously described (Perez *et al.*, 2008). Briefly, genomic DNA was extracted from modified and control cells using the MasterPure™ DNA purification kit (Epicentre Biotechnologies, Madison, WI). After radioactive polymerase chain reaction (PCR) amplification of the region encompassing the CCR5 ZFN target region, the Surveyor nuclease (Surveyor mutation detection kit; Transgenomic, Omaha, NE) was used according to the manufacturer. The following primers were used in the PCR: CCR5: 5′-AAGATGGA TTATCAAGTGTCAAGTCC-3′ and 5′-CAAAGTCCCACT GGGCG-3′; CCR2: 5′-GGCTCTACTCGCTGGTGTTC-ATC-3′ and 5′-GGCAGAGTAATAAGAAAAAGCAGATC-3′; and ABLIM2: 5′-GGGTTCTCTTGTTTGTTCCATACC-3′ and 5′-GGGTCAGTCTCTTAGCCGTGTG-3′. Products were resolved by PAGE and bands quantified by phosphorimaging.

T-cell repertoire by T-cell receptor Vβ analysis

Approximately 1×10^6 cells from Day 0 (before transduction) and various time points post-transduction and expansion were collected, and cells were pelleted and frozen. The pellets were analyzed by TCLand Expression (Nantes, France) for the expression of 26 TCR Vβ chain genes. Complementary determining region 3 length distribution (CDR3-LD) alteration was performed on a AB3730 capillary sequencer and analyzed using GeneMapper® and Immunoscope® software (Pannetier *et al.*, 1995). Global CDR3-LD alterations were measured according to Gorochov *et al.* (1998). T-cell receptor (TCR) variable chain Vβ to hypoxanthine-guanine phosphoribosyltransferase transcript (HPRT) ratios were measured by real-time PCR (Gagne *et al.*, 2000). Data were displayed as a bidimensional TopView or as a tridimensional TCLandscape® (Sebille *et al.*, 2001; Guillet *et al.*, 2002) where the x-axis displays the 26 Vβ families, the z-axis shows the Vβ transcript/HPRT transcript ratios, and the y-axis gives the 13 possible CDR3 lengths per Vβ family. Percentages of alterations compared to healthy donors as controls are represented as a color code, ranging from deep blue (values −30%) to dark red (30%) in the integrated landscapes (Sebille *et al.*, 2001; Guillet *et al.*, 2002). MatLab® 5.3 software (The MathWorks Inc, Natick, MA) was used to compute and display the data.

Karyotyping

Karyotype analysis was performed by the University of Pennsylvania Clinical Cytogenetics Laboratory, which is accredited by the College of American Pathologists. Colcemid as a metaphase arresting agent was added to cell cultures for 2 hr. Cells were exposed to hypotonic solution for 40 min at 37°C, followed by three changes of 1:3 glacial acetic acid and methanol. Cell suspensions were dropped onto water-wet microscope slides. Dried slides were aged in a 60°C oven for 14 hr and stained with Wright Stain for G-banding and analyzed at 100x magnification on a bright field microscope. Images were captured and karyotypes prepared on the Applied Imaging Computer Karyotyping system. Three slide preparations were made from each condition, and a total of 10–15 cells were analyzed per slide.

In vivo *toxicity studies in mice*

NOD/*scid*/γ_c^{null} (NSG) mice (Shultz *et al.*, 2005) were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed in microisolator cages under sterile conditions. While xenogeneic graft-versus-host disease (xGVHD) drives activation and expansion of human T cells in this model (Garcia *et al.*, 1997), it also leads to xGVHD-mediated death in host animals within 2–4 months, depending on infused cell dose and route of administration. T-cell expansion amplifies detection of any genotoxic event, which partially mitigates concern regarding the ability of this model to detect such events within the limited study duration. All animal-use protocols were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Studies used 4–6-week-old male and female mice in equal proportions. Including a pilot study that served to validate the design for Study 1, four unique human donors overall were used for *in vivo* studies, human T cells were collected, and CCR5-ZFN modified, expanded, and cryopreserved as described above. Twenty million T cells in a cushion of 2 million autologous PBMC (to promote engraftment) were administered to each of the 24 mice per donor in the CCR5-ZFN-modified T-cell group and eight mice per donor in the unmodified T-cell control group. A dose of 20 million cells in a mouse corresponds to a human dose of $\sim5\times10^{10}$ cells. The total dose of CCR5-ZFN-modified T cells administered during preclinical studies from four separate human donors was $>3\times10^9$ cells, which approaches a single human dose of $5–10\times10^{10}$ total cells. Cells were injected via the tail vein to mimic the clinical route of administration. Animals were weighed at baseline, weekly post-treatment, and prior to necropsy. Peripheral blood was collected by retro-orbital bleeding at 2–4-week intervals. Daily cage-side observations were performed to assess gross animal health. Twice weekly, animals were physically examined and formally scored for signs of xGVHD, including but not limited to activity level, behavior, general grooming, and condition of fur and posture.

Animals judged to be suffering from severe xGVHD were euthanized.

All animals were scheduled to undergo a full necropsy according to a schedule outlined in the experimental design. However, animals judged to be suffering from severe xGVHD were euthanized and necropsied before their scheduled sacrifice date. Peripheral blood collected by cardiac puncture was analyzed for the presence of human cells using the TruCount assay (BD Biosciences, Franklin Lakes, NJ). Samples were stained for human CD45, CD4, and CD8. Animals were scored as positively engrafted if there were greater than 20 $CD45^+$ cells/uL of peripheral blood. Bone marrow harvested from femurs was also stained with antibodies to human CD45, CD4, and CD8, and analyzed by flow cytometry. A second study was conducted utilizing 10 million cells per animal administered intravenously, in the absence of a PBMC cushion with the aim of minimizing xGVHD and supporting longitudinal observation of the animals to detect potential delayed adverse effects. In this study, IL-2 intraperitoneal injections were given at 2000 IU biweekly at days 0, 4, 7, and 10. There was a single donor for this study and all 38 animals received CCR5-ZFN-modified T cells.

Multiple organs were subjected to gross macroscopic analysis at necropsy in order to detect major changes that may have resulted from tumor formation or xGVHD-induced gross tissue damage. Organs and tissues collected included brain, heart, kidney, liver, spleen, lungs, lymph nodes (typically absent), skin (ear), reproductive organs, eye, skeletal muscle, thymus (typically absent), and gut. Any unusual or unidentified organs or tissues were also collected. The brain, heart, kidney, liver, lung, testes (if present), and spleen were weighed. All collected organs were sectioned: one section was formalin fixed and the other was snap-frozen. Fixed tissues were used to prepare paraffin blocks for H&E analysis and immunohistochemistry. In rare cases where potential neoplasms were detected, sections of the affected tissue were stained for with anti-human-CD3 mAb to determine the T-cell content of the neoplasm. Lymphoid neoplasms characteristic of B-cell lymphoma were identified in a pilot study in both control and treatment groups. There was an absence of CD3 staining, and blinded independent histopathologic analysis, performed by Pathology Associates International (PAI, Frederick, Maryland), determined that the neoplasms were characteristic of B-cell lymphoma. Additional review of the histopathology findings in studies 1 and 2 was obtained by a board certified hematology/oncology pathologist at the Hospital at the University of Pennsylvania and also by WIL-Biotechnics LLC (Hillsborough, NC). Retrospective analysis of the donors showed both were Epstein-Barr-virus (EBV) positive. Therefore, the tumors may have arisen from B cells in the PBMC cushion. These cells are known to result in lymphoproliferation in immune-deficient mice (Mosier *et al.*, 1992; Wagar *et al.*, 2000), which illustrates the sensitivity of this model.

Engraftment of human CD4 T cells in skin, liver, testes, lung, ovaries, brain, bone marrow, kidneys, heart, spleen, and blood was assessed by duplex PCR involving coamplification of human and mouse repetitive sequences from snap-frozen tissue sections (Pelz *et al.*, 2005). The limit of detection of the assay was 1 copy of human DNA per 2000 copies of mouse genomic DNA. Quantitation of CCR5-ZFN-modified T cells in blood, spleen, and lung tissues was performed using the Cel-1 Surveyor nuclease assay described above and previously (Perez *et al.*, 2008).

Statistical analysis in vivo *toxicity studies*

The data from *in vivo* toxicity studies consisted of up to nine observations (baseline plus eight weekly follow-ups) on animals in two treatment arms (mock transfected and SB 728-T transfected). Two experiments were conducted, each with three separate T-cell donors (although one donor was used in both experiments). To account for this, we considered each donor-experiment combination a group, leading to six groups. We analyzed the weight data using mixed linear models incorporating group, treatment arm, week, and animal as explanatory factors. All analyses were conducted in Proc Mixed of SAS Version 9.2 (SAS Institute, Cary, NC) on a Sun server.

Results

Enhanced efficiency of zinc finger nuclease (ZFN) delivery by Ad5/F35 vector following CD3/CD28 stimulation

We have previously demonstrated that activation of T cells via immobilized anti-CD3 and anti-CD28 mAbs enabled highly efficient and potent stimulation of T cells compared with either phorbol 12-myristate 13-acetate (PMA) or PHA activation (Levine *et al.*, 1995, 1997). Furthermore, since 1996 the CD3/CD28 activation method has been used to prepare several hundred activated T-cell products that have been infused into study subjects in clinical trials (Levine *et al.*, 2002, 2006; Laport *et al.*, 2003; Rapoport *et al.*, 2004, 2005, 2009; Porter *et al.*, 2006). *In vitro* studies performed with the ZFNs encoded by SB-728 demonstrated that transient ZFN expression post gene transfer using a chimeric Ad5/F35 adenoviral vector was sufficient to modify >30% of the *CCR5* alleles (Perez *et al.*, 2008; and data not shown). Ad5/F35 vector had previously been demonstrated to efficiently transduce hematopoietic cells, including T lymphocytes (Shayakhmetov *et al.*, 2000; Chen *et al.*, 2002; Cho *et al.*, 2003; Nilsson *et al.*, 2004; Schroers *et al.*, 2004; Jung *et al.*, 2005; Lu *et al.*, 2006). Earlier studies by other groups in which T lymphocytes had been transduced with Ad5/F35 vectors used PHA or PMA for cell stimulation (Chen *et al.*, 2002; Schroers *et al.*, 2004). However, the use of either PMA or PHA, available in research grades only, is problematic from a regulatory and toxicity perspective. We directly compared the efficiency of Ad5/F35 transduction in experiments with two separate Ad5/F35 vectors encoding either GFP (Fig. 1A) or a ZFN targeted to *CCR5* (Fig. 1B) following PHA stimulation or anti-CD3/CD28 stimulation of T cells. CD3/CD28-stimulated cells were transduced more efficiently, as measured by both percent GFP positive cells and GFP mean fluorescence intensity (Fig. 1A). Using the CCR5 ZFN previously selected for further clinical development (Perez *et al.*, 2008), CD3/CD28 stimulation of T cells also facilitated superior genetic modification of *CCR5* compared with PHA stimulation as shown in Figure 1B and Table 1. We have previously shown that during the first 5–7 days post-stimulation, T-cell expansion mediated by PHA, PMA, and CD3/CD28 stimulation is roughly equivalent. However, further expansion and restimulation demonstrated the superiority of

CD3/CD28 stimulation in terms of cell yield and cell function (Levine *et al.*, 1997). Taken together, these data, and the regulatory advantage of using a clinically validated method for T-lymphocyte stimulation, indicated that maximal ZFN-mediated *CCR5* disruption and T-cell yield could be obtained by adapting the Ad5/F35 delivery of ZFNs to large-scale CD3/CD28 cell stimulation to generate sufficient numbers of *CCR5*-modified T cells for a clinical trial.

Development of clinical ex vivo *transduction process*

While early experiments showed that Ad5/F35-mediated transduction of CCR5-ZFNs was efficient, these studies were conducted with a media formulation containing fetal calf serum (Perez *et al.*, 2008). This presented a regulatory obstacle for clinical process development due to the risk of bovine spongiform encephalopathy (BSE) contamination. Of more concern is the reported immune reactivity to adoptively transferred cells prepared with bovine serum, even in immunosuppressed subjects, ranging from anti–fetal calf serum (FCS) antibodies to Arthus reactions and anaphylaxis (Selvaggi *et al.*, 1997; Mackensen *et al.*, 2000; Horwitz *et al.*, 2002; Tuschong *et al.*, 2002). We first performed Ad5/F35 transduction in standard clinical media that we previously validated for T-cell expansion. This medium was based on XVIVO-15, developed for serum-free cultures, but it was supplemented with pooled human AB serum as used in several previous clinical trials, including gene transfer trials (Levine *et al.*, 2002, 2006; Laport *et al.*, 2003; Rapoport *et al.*, 2004, 2005, 2009; Porter *et al.*, 2006). This media was compared to the research-grade media consisting of RPMI 1640 supplemented with 10% FCS. While there was efficient transduction in RPMI 1640/10% FCS, there was minimal transduction in XVIVO-15 supplemented with 5% pooled human serum (data not shown), likely due to anti-adenovirus antibodies in lots of pooled human serum (Nwanegbo *et al.*, 2004). We then tested a medium formulation of XVIVO-15 supplemented with 0.5% human serum albumin without addition of pooled human serum. To optimize T-cell expansion following transduction, medium supplemented with pooled human serum was added 3 days after the addition of Ad5/F35 CCR5-ZFN vector. As shown in Figure 2, efficient transduction was obtained when compared with pooled human serum–supplemented media.

Using the transduction media and process described above, we next established the optimal Ad5/F35 CCR5-ZFN vector MOI to maximize ZFN-mediated *CCR5* modification while minimizing the potential negative effects of higher levels of vector or ZFN on T-cell expansion. Additionally, it was important to establish whether off-target gene modification could be observed at higher MOI. Based on pilot studies, we focused on a range of MOI from 400 to 2,000 infectious units per cell, which at our observed particle to infectious unit ratio of 8–10 corresponds to 3,200 to 20,000 vector genomes per cell. As expected, Ad5/F35 CCR5-ZFN transduction has a negative impact on overall T-cell expansion capacity at higher MOIs in both healthy donors and HIV-1 infected donors. As shown in Figure 3A in an HIV-1 infected donor, a modest impairment of T-cell growth was observed at MOIs 800, 1200, and 2000, with no, or minimal, effect on T-cell growth at MOIs of 400 and 600. A similar effect was observed in an identical MOI titration with a healthy donor (data not shown). To establish the effect of Ad5/F35 CCR5-ZFN vector MOI on the frequency of both on- and off-target gene modification, samples from this experiment were analyzed for disruption of the *CCR5* locus, as well as disruption at two potential off-target sites, *CCR2* and *ABLIM2*, previously identified by a bioinformatic search of the human genome (Perez *et al.*, 2008). On- and off-target modification was determined by Surveyor nuclease assay and deep sequencing of the modified cells as described (Perez *et al.*, 2008). Figure 3B shows that at Day 6 post-transduction, there was a trend for increased *CCR5* modification with increasing MOI from 400 to 800, with no added benefit from higher MOIs. By Day 10 of culture, only marginal increases in *CCR5* disruption were observed at higher MOIs. For *CCR2*, low level disruption (<4%) was observed at an MOI of 400, but the frequency of disruption did not increase with higher MOI, and by Day 10 there was a relative decrease in the percentage of *CCR2* disrupted cells. There was no observed disruption at *ABLIM2* at a level of detection in the Surveyor nuclease assay of ~1% (data not shown).

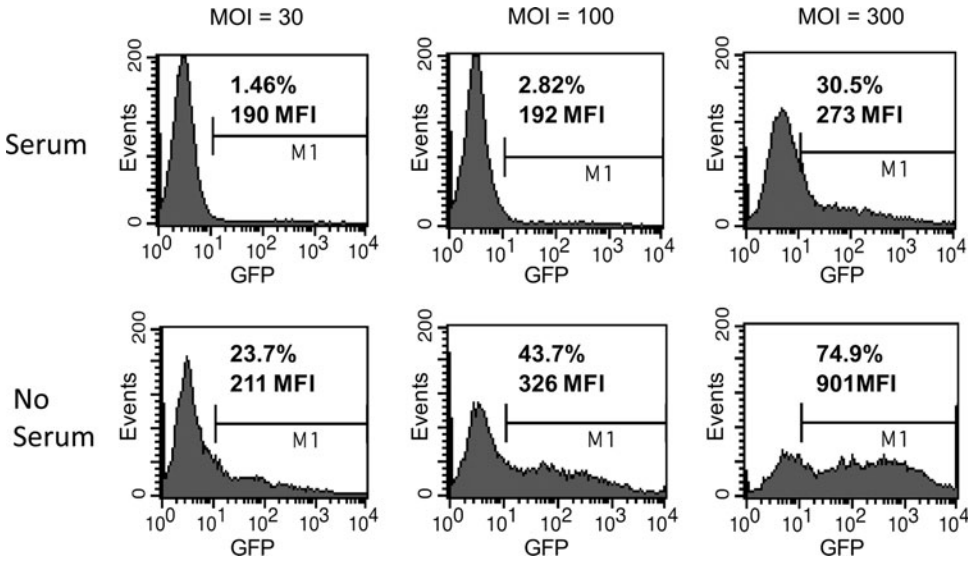

FIG. 2. Human serum inhibition of Ad5/F35 transduction in T cells. Primary CD4 T cells were activated overnight with anti-CD3/28 antibody-conjugated beads at a 3:1 bead: cell ratio and then transduced at an MOI of 30, 100, or 300 with Ad5/F35 vector-expressing GFP in the presence of 5% pooled human serum or in the absence of human serum. GFP was measured on Day 3 after initial activation. The percent of cells expressing GFP and the MFI of cells gated as positive are shown.

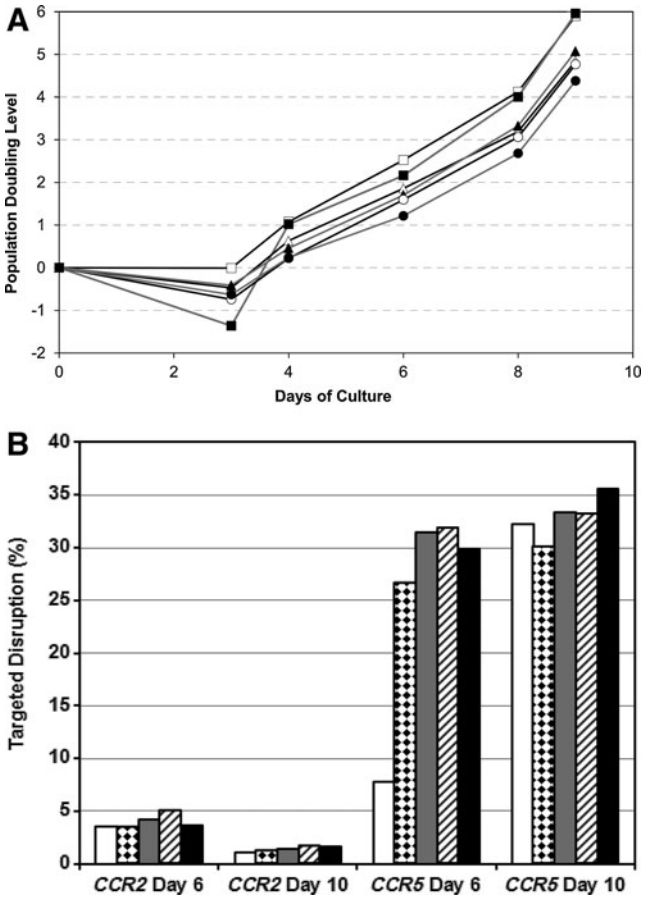

FIG. 3. Ad5/F35 CCR5 ZFN vector titration and T-cell expansion. To evaluate the best balance of MOI for efficient transduction and preservation of robust cell expansion, CD4 T cells were activated overnight with CD3/28 beads as previously described and then transduced with Ad5/F35 vector-encoding CCR5-ZFN at MOI's of 0 or mock transduced, 400, 600, 800, 1200, and 2000. **(A)** Cells were monitored for growth and population doubling level calculated based on starting number of cells and number of cells continued in culture at each time point after sampling: Mock transduced (black line, open square symbols), MOI 400 (gray line, filled square symbols), MOI 600 (black line, open triangle symbols), MOI 800 (gray line, closed triangle symbols), MOI 1200 (black line, open circle symbols), and MOI 2000 (gray line, closed circle symbols). **(B)** Gene disruption at *CCR5* and the closely related *CCR2* was measured by the SURVEYOR nuclease assay: MOI 400 (white), MOI 600 (diamond hatch), MOI 800 (gray), MOI 1200 (diagonal hatch), and MOI 2000 (black).

Previous studies conducted in small scale cultures using ultra-deep sequencing showed only rare (~1/20,000) events at *ABLIM2* (Perez *et al.*, 2008). Based on these results, and to minimize the effect of higher MOI on cell growth, an MOI of 600 was chosen for further clinical development.

Functional and phenotypic comparability of CCR5-ZFN-modified and -unmodified T cells

We previously developed a large-scale method of T-cell isolation and culture in static gas permeable bags, followed by washing, concentration, and formulation of the cell product sufficient to meet doses of expanded T cells of up to 3×10^{10} (Levine *et al.*, 1998). More recently, we have integrated a dynamic bioreactor (Wave, GE Healthcare Life Sciences, Piscataway, NJ) that allows for media perfusion during the culture process (Hami *et al.*, 2004; Hollyman *et al.*, 2009). A possible advantage of the dynamic perfusion method is that it achieves further reduction of remaining adenoviral vectors that could contribute to the immunogenicity of the Ad5/F35-transduced T-cell product. Translating the research scale static process to a clinical scale dynamic perfusion culture required validation studies that addressed T-cell growth, possible decreases in transduction efficiency, or dilution of autocrine T-cell cytokine production by perfusion. Deficiencies in any of these processes could result in less potent T cells as the final product. To address these questions, we first compared growth rates in untransduced and transduced research scale T-cell cultures to growth rates in transduced clinical scale cultures employing the dynamic perfusion system. Shown in Figure 4A is a representative growth curve comparing these conditions using cells from an HIV-1 infected donor. These data indicate transduced cells were more efficiently expanded using the clinical scale dynamic perfusion procedure than the static small-scale procedure. In fact, growth of transduced cells using the clinical scale dynamic perfusion system was comparable to growth of untransduced cells using the static small-scale procedure. While it appears in Figure 4A that the growth rate accelerated in the clinical scale condition between Day 8 and Day 9, this likely reflects a sampling artifact resulting from the design of the bioreactor bag. On Day 9, the cell count was obtained from a sample after all cells had been removed from the bioreactor bag and thus more accurately reflects the total number of cells in the culture. In addition, the clinical scale process enabled targeted ZFN-mediated *CCR5* disruption at 20.9% of alleles on Day 9, compared to 14.9% of alleles for the research scale process.

As a gauge of T-cell function, the production of IL-2, TNF-α, and Interferon-γ was measured by the cytometric bead array assay in supernatants from research scale untransduced, research scale CCR5-ZFN transduced, and clinical scale CCR5-ZFN transduced cultures (Fig. 4B). Expanded T cells from the HIV-1 infected donor shown in Figure 4A were harvested, and the remaining anti-CD3/CD28 mAb-coated beads were removed. The cells were washed, resuspended in fresh media, and restimulated with fresh anti-CD3/CD28 mAb-coated beads. Supernatants were collected 24 hr later. All three culture methods resulted in comparable production of IL-2, TNF-α, and Interferon-γ, indicating that CCR5-ZFN-modified T cells transduced at research scale or at clinical scale following media perfusion in the wave bioreactor were not impaired in cytokine production compared to control untransduced T cells. These results were confirmed in a subsequent clinical scale run with a separate HIV-1+ donor in which production of granulocyte macrophage colony-stimulating factor (GM-CSF) and chemokines was also examined (Fig. 4C). Chemokine production, like cytokine production, is an important functional indicator in the setting of HIV infection (Verani and Lusso, 2002). Spontaneous secretion of cytokines and chemokines (production in the absence of restimulation) was also measured, as this might better

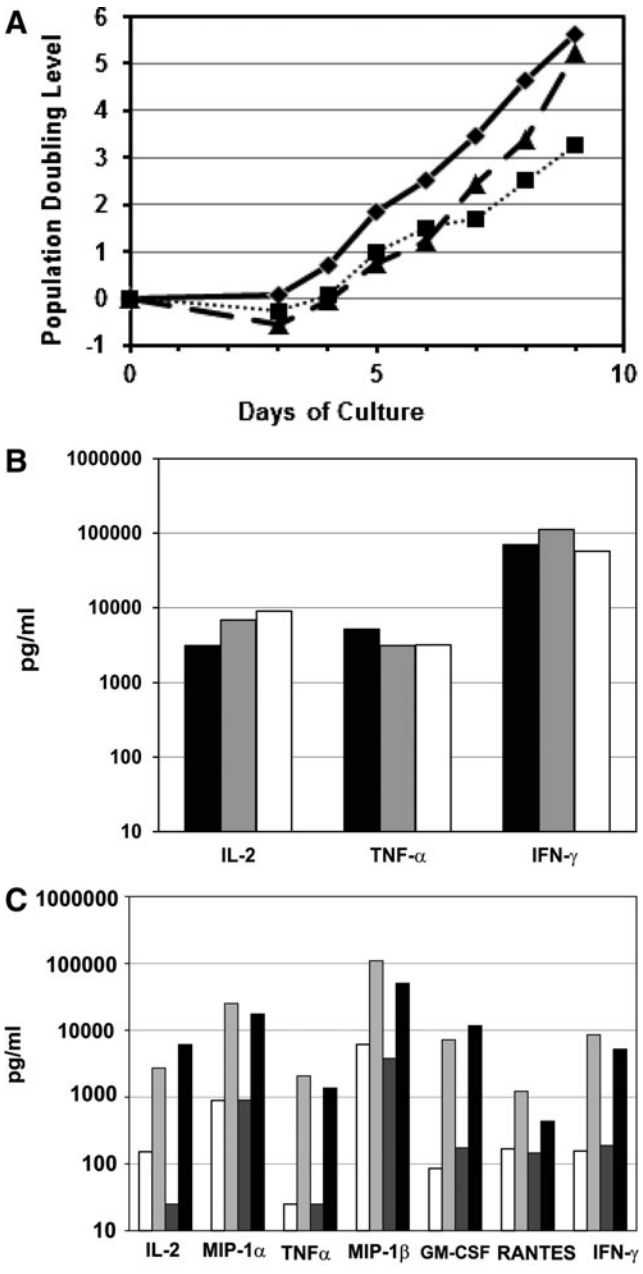

FIG. 4. Validation of large-scale *ex vivo* clinical process for T-cell expansion and CCR5 modification incorporating a dynamic perfusion bioreactor. **(A)** Cells from an HIV-1 infected donor were stimulated *ex vivo* and transduced as described in the Methods section. Research scale untransduced, solid line and diamond symbols; research scale transduced, dotted line and square symbols; clinical scale transduced, dashed line and triangle symbols. **(B)** The production of IL-2, TNF-α, and Interferon-γ in supernatants are shown—research scale untransduced, research scale CCR5-ZFN transduced, and clinical scale CCR5-ZFN-transduced cultures. Expanded T cells from the culture in **(A)** were harvested, washed, resuspended in fresh media, and restimulated with fresh anti-CD3/CD28 mAb-coated beads. Supernatants were collected 24 hr later. Research scale untransduced, black bars; research scale transduced, gray bars; clinical scale transduced, white bars. An additional HIV-1 infected donor **(C)** was compared for production of cytokines as well as β-chemokines as in **(B).** Spontaneous secretion of cytokines and β-chemokines (production in the absence of restimulation) was also assayed as a measure potency of final product cells for infusion. Cells were harvested at the end of culture, and beads were removed and resuspended in fresh culture media. Supernatants were collected 24 hr later. Shown is the production of IL-2, TNF-α, MIP-1α, MIP-1β, GM-CSF, RANTES, and Interferon-γ. Research scale untransduced and unstimulated (spontaneous), white; research scale untransduced and restimulated with fresh anti-CD3/anti-CD28 mAb beads, light gray; clinical scale transduced and unstimulated (spontaneous), dark gray; clinical scale transduced and restimulated with fresh anti-CD3/anti-CD28 mAb beads, black.

reflect the functional status of the final cell product upon reinfusion to study subjects. Figure 4C depicts production of IL-2, TNF-α, MIP-1α, MIP-1β, GM-CSF, RANTES, and Interferon-γ by research scale untransduced control T cells and clinical scale CCR5-ZFN-transduced T cells. Spontaneous secretion of cytokines was comparable between the two conditions for all cytokines except for IL-2. Spontaneous IL-2 levels were lower in the clinical scale culture, which might reflect increased consumption in a rapidly growing culture. Perfusion culture media is supplemented with IL-2, but media used to assess spontaneous cytokine production was not supplemented with IL-2. For cells restimulated with fresh anti-CD3/CD28 mAb-coated beads, production of all cytokines and chemokines tested was comparable between the control research scale condition and the clinical scale transduced CCR5-ZFN-modified condition. Taken together, these results demonstrate functional comparability in control T cells and CCR5-ZFN-modified T cells expanded under clinical scale media perfusion conditions.

Control unmodified T cells and transduced CCR5-ZFN-modified T cells were also phenotyped by flow cytometry. Equivalent levels of $CD3^+CD4^+$, $CD4^+CD28^+$, activation (CD25), and homing (CD62L) receptors were observed on cells expanded by either method. $CD3^+CD4^+$, $CD3^+CD28^+$, and $CD3^+CD25^+$ percentages were routinely >90% (data not shown) and consistent with previous studies (Levine *et al.*, 1996, 1998). To look for putative chromosomal abnormalities that might be induced by CCR5-ZFN modification of T cells, the FDA and the NIH Recombinant DNA Advisory Committee (RAC) requested that we perform karyotype analysis, in addition to the bioinformatic search, and ultra-deep sequencing of potential CCR5-ZFN off-target sites, and intranuclear staining for sites of DNA double-strand breaks conducted in our previous study (Perez *et al.*, 2008). In both control expanded and CCR5-ZFN modified and expanded T cells, a normal karyotype was described and no clonal abnormalities were present (data not shown).

TCR repertoire analysis can provide information on the loss of diversity of CD4+ T cells during progressive HIV infection (Connors *et al.*, 1997; Gea-Banacloche *et al.*, 1998, 2000; Gorochov *et al.*, 1998; Martinon *et al.*, 1999). TCR analysis can detect aberrant clonal expansions or deletions (Pannetier *et al.*, 1995) and is thus essential for safety monitoring in adoptive T-cell transfer therapy. For example, TCR repertoire analysis has been used to track the development of leukemic clones in children after gene therapy for x-linked severe combined immunodeficiency (SCID-X1) (Hacein-Bey-Abina *et al.*, 2003). T cells from a total of three donors (one

healthy, two HIV-1+) were analyzed for TCR Vβ chain repertoire before and after a control research scale expansion or after a clinical scale expansion of CCR5-ZFN-transduced cells. Figure 5 illustrates a three-dimensional plot of the complementary determining region 3 (CDR3) length distribution for the 26 TCR Vβ family genes analyzed as a ratio of the Vβ to HPRT transcripts. Similarity was seen between the research scale untransduced condition and the clinical scale CCR5-ZFN-modified condition in the average percent perturbation as measured by skewedness to healthy donor controls (Supplementary Table 1). Thus, by multiple *in vitro* measures, control, and transduced CCR5-ZFN-modified T cells in both healthy and HIV+ donors were equivalent in phenotype, expansion, cytokine and chemokine production, and TCR diversity.

In vivo *toxicity studies in mice*

In pre–investigational new drug (IND) discussions, the FDA requested that animal studies be performed to evaluate the safety of CCR5-ZFN-modified T cells. Although there are limitations, immunodeficient mouse xenotransplant models can address some safety concerns by using *in vivo* engraftment to search for evidence of genotoxicity. The NOD/*scid*/$_{\gamma}$c$^{\text{null}}$ (NSG) mouse (Shultz *et al.*, 2005) supports high levels of engraftment of activated human T cells. In addition to lacking T-, B-, and natural killer cells, NSG mice also have impaired dendritic cell function (Ito *et al.*, 2002). While xenoreactivity drives activation and pauci-clonal expansion of human T cells in NSG mice (Garcia *et al.*, 1997), it also leads to xenogeneic graft-versus-host disease (xGVHD)-mediated death in host animals (Nervi *et al.*, 2007). Consequently, we performed a series of pilot animal studies to establish the optimal cell dose, route of administration, and study duration in the NSG mouse model. FDA feedback following these pilot studies was incorporated into the design of full-scale studies 1 and 2 shown in Figure 6 and described below. These studies were conducted to evaluate whether *ex vivo* CCR5-ZFN modification of T cells led to *in vivo* T-cell proliferative abnormalities or tumor formation related to the CCR5-ZFN-modified T cells.

In the first study, 20 million CCR5-ZFN-modified CD4 T cells compared to mock control unmodified cells were administered along with 2 million autologous PBMCs (Table 2) for each of three unique human donors. Engraftment of human cells was detected overall across donors in 92–100% of animals. There was no adverse effect on CD4 T-cell engraftment of the CCR5-ZFN-modified cells compared to mock control unmodified cells (Supplementary Fig. S1). Peak engraftment levels were detected at 8 weeks post injection, corresponding with the development of xGVHD, which is endemic to this model (Nervi *et al.*, 2007). There was also no adverse effect on body weight of the CCR5-ZFN-modified cells compared to mock control unmodified cells for each of three unique human donors, indicating no global adverse effects of the CCR5-ZFN-modified cells (Supplementary Fig. S2). Study 2 was performed with a single arm of CCR5-ZFN-modified cells and without a PBMC cushion to reduce xGVHD and thereby to extend the window for monitoring tumor formation. In this study, animals were evaluated for up to 18 weeks due to development of xGVHD across all animals prior to the planned 6-month duration.

In each of the two animal studies, comparison of *CCR5* gene modification levels in the initial cell product compared to the cells isolated from the spleen at necropsy indicated no selective outgrowth of CCR5-ZFN-modified cells *in vivo* (Table 2), confirming prior observations (Perez *et al.*, 2008). Microscopic histopathology in each of the studies and groups was consistent with xGVHD. No CCR5-ZFN- or T-cell-related tumor formation was observed in any animals. All unscheduled deaths where necropsy could be performed were also confirmed to be xGVHD-associated. The number of unscheduled deaths was similar between the mice

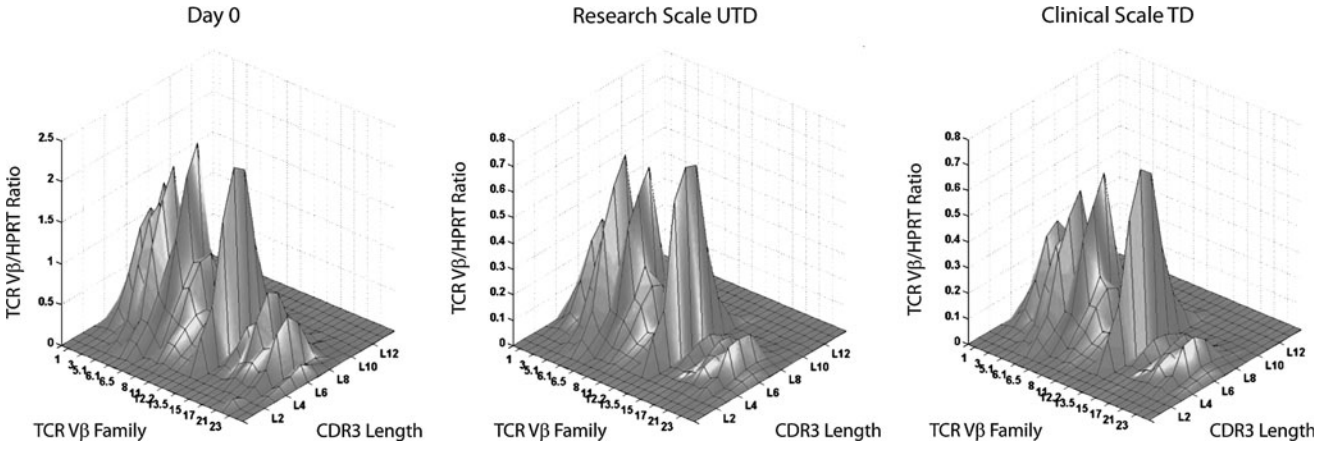

FIG. 5. T-cell receptor (TCR) Vβ repertoire of CCR5-ZFN T cells produced with large-scale *ex vivo* clinical process. TCR Vβ chain repertoire before and after a control research-scale expansion or after a clinical-scale expansion of CCR5-ZFN-transduced cells from a representative HIV+ donor. T cells from a total of three donors (one healthy, two HIV+) were analyzed. Three-dimensional plot of the complementary determining region 3 (CDR3) length distribution for the 26 TCR Vβ family of genes was analyzed as a ratio of the Vβ to hypoxanthine-guanine phosphoribosyltransferase transcript (HPRT). X-axis, TCR Vβ family; y-axis, TCR Vβ /HPRT ratio; z-axis, CDR3 length. Calculated TCR Vβ percentages of alteration are listed in Supplementary Table 1.

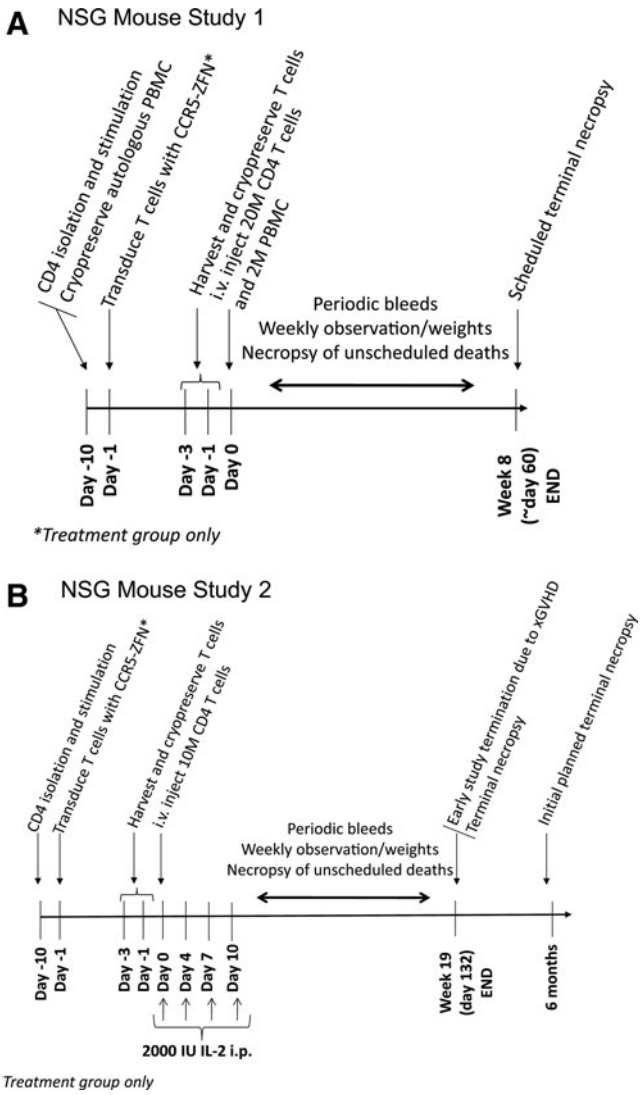

FIG. 6. NOD/*scid*/γ_c^{null} (NSG) mouse–human xenograft biotoxicity study design. The days on study and the procedures at each timepoint are shown. See Methods for a detailed description of study procedures. **(A)** Study 1 utilized three unique human donors. CD4 T cells were transduced (24 mice/donor) or mock transduced (8 mice/donor) with Ad5/F35 vector-encoding CCR5-ZFN, and infused along with autologous peripheral blood mononuclear cells (PBMC) to support engraftment. Study 1 duration was restricted to 8 weeks due to onset of xenogeneic graft-versus-host disease (xGVHD). **(B)** Study 2 (38 mice) was performed using a single donor and without co-injection of autologous PBMC to delay xGVHD onset and allow a longer study observation period. Engraftment was supported by IL-2 injections concomitant with CCR5-ZFN-modified CD4+ T cells.

receiving CCR5-ZFN-modified T cells and control mice (Table 2). Therefore, by all criteria agreed upon in consultation with the FDA (clinical observations, gross observations upon necropsy, molecular studies, and histopathology) no CCR5-ZFN-related toxicity was detected in any of the studies. The *in vivo* studies complemented *in vitro* studies performed in both healthy and HIV-1+ donors (Supplementary Table 2). Data from these *in vivo* and *in vitro* studies were submitted to the FDA to support the first in human clinical trial evaluating

TABLE 2. *IN VIVO* STUDIES—UNSCHEDULED DEATHS, *CCR5* MODIFICATION IN THE INFUSED CELLS AND RECOVERED CELLS, CHANGE IN BODY WEIGHT, GRAFT-VERSUS-HOST DISEASE, AND PATHOLOGY FINDINGS

Study	*Donor*	*Group*	n	*Unscheduled deaths*	*CCR5 modification pre-infusion*	*CCR5 modification at necropsy*[a] *mean ± SD*	*Avg % weight change ± SD*	*% GVHD*[b]	*Pathology findings*
1	10	CCR5-ZFN	24	18 (75%)	27.5	0.7 (1.2)	−5.8 (6.2)	33.3	None detected
1	10	Mock	8	8 (100%)	0	0	NA	37.8	None detected
1	11	CCR5-ZFN	24	0	24.8	2.3 (4.0)	−3.7 (3.9)	4.2	None detected
1	11	Mock	8	0	0	0	−4.6 (4.0)	12.5	None detected
1	12	CCR5-ZFN	24	3 (13%)	20	0	−5.4 (6.7)	0	1) Hepatic adenoma 2) Liver and kidney nodules, no abnormal histopathology
1	12	Mock	8	1 (13%)	0	0	−6.1 (3.7)	0	Tubular degeneration in testes with epididymis
2	13	CCR5-ZFN	38	38 (100%)[c]	36.1	25.9 (6.9)	−8.5 (1.0)	100	None detected

[a]Post-measurement taken upon terminal necropsy from spleen in animals with high persistence of human DNA as measured by PCR.
[b]xGVHD was determined by the presence of 2 of 3 clinical observations (activity, fur, posture).
[c]Study terminated on Day 132 due to development of xGVHD across all animals prior to planned 6-month study date.
ZFN, zinc finger nuclease; GVHD, graft-versus-host disease; NA, not applicable—no animals survived to study termination date due to GVHD.

CCR5-ZFN-modified T cells in HIV-1 study subjects (www.clinicaltrials.gov identifier number NCT00842634).

Discussion

We show here an integrated strategy for the scale-up, validation, and provision of safety data to regulatory authorities for the first trial of *ex vivo* zinc finger nuclease genome-edited cells. Sufficiently large numbers of modified cells can be generated using clinically compatible procedures and reagents, and these modified cells retain functional activity and phenotypic properties comparable to research scale methods. We have previously shown that highly efficient and potent activation of T lymphocytes is achieved following costimulation with anti-CD3/CD28 beads when compared to either PHA or PMA activation through protein kinase C (Levine *et al.*, 1995, 1997). Moreover, while the T-cell expansion rate mediated by PHA, PMA, and anti-CD3/CD28 stimulation is roughly equivalent during the first 5–7 days poststimulation, only costimulation via anti-CD3/CD28 enables extensive expansion of functional T cells secreting high levels of cytokines and chemokines (Levine *et al.*, 1997). We show that this anti-CD3/CD28 costimulatory activation method produces an expanded T-cell product with enhanced Ad5/F35 transduction efficiency and superior genetic modification, using either the GFP or ZFN-CCR5 Ad5/F35 vectors tested. In addition, while earlier work by others utilized PHA- or PMA-stimulated T cells for Ad5/F35 vector transduction (Chen *et al.*, 2002; Schroers *et al.*, 2004), both PHA and PMA pose regulatory and toxicity challenges for clinical use.

Chimeric Ad5/F35 adenoviral vector derived from swapping the knob region of the fiber protein results in a pseudotyped virus with modified tropism and allows for transduction of cells of the hematopoietic lineage (Shayakhmetov *et al.*, 2000; Chen *et al.*, 2002; Hurez *et al.*, 2002; Cho *et al.*, 2003; Leen *et al.*, 2004; Nilsson *et al.*, 2004; Schroers *et al.*, 2004; Jung *et al.*, 2005; Lu *et al.*, 2006). While the efficiency of Ad5/F35 delivery of CCR5-ZFNs to T cells is attractive, a potential drawback is the presence of residual adenoviral vector at the end of production. Though our clinical process incorporates perfusion during culture and extensive washing at the end of culture and prior to formulation, adenoviral vector and residual ZFN antigens theoretically could contribute to generation of an adenovirus immune response, which could limit administration of multiple infusions of these cells (Nwanegbo *et al.*, 2004). One way to avoid this issue is to use alternative methods of delivery such as RNA electroporation, recently been shown to effectively express functional gene constructs *in vivo* (Zhao *et al.*, 2010).

Modification of culture media was necessary for efficient clinical scale *ex vivo* transduction with the adenoviral vector Ad5/F35. Due to the presence of neutralizing adenoviral antibodies in pooled human serum, reformulation of media supplements to contain human serum albumin in substitution for pooled human serum was shown to enhance transduction of Ad5/F35 CCR5-ZFN vector efficiency with minimal impact on *ex vivo* expansion. Ad5/F35 CCR5-ZFN transduction at higher MOIs resulted in reduced T-cell growth in both healthy donors and HIV+ donors compared to the overall T-cell expansion achieved at lower MOIs.

Two potential off-target sites, *CCR2* and *ABLIM2*, previously identified by a bioinformatics search of the human genome (Perez *et al.*, 2008), were monitored in this study. Importantly, the low level of disruption (<4%) observed at *CCR2* did not increase with higher vector MOIs at Day 6 and decreased at Day 10. This could be due to deleterious effects of higher adenovirus vector exposure or higher levels of ZFNs. It may be that multiply transduced *CCR5*- and *CCR2*-modified T cells are at a growth disadvantage through vector-mediated or other effects. In humans, mutant alleles of *CCR2* have been correlated with delayed progression to AIDS in HIV-infected individuals, suggesting a possible beneficial phenotype from *CCR2* loss in HIV-infected individuals (Smith *et al.*, 1997). The next off-target site identified (Perez *et al.*, 2008), *ABLIM2* is located in an intron, and we show here that there was no detected disruption at *ABLIM2* using the clinical cell-processing methods described. A newer unbiased method for analysis of ZFN specificity has identified a restricted number of additional off target cleavage sites under conditions of ZFN overexpression in K562 cells (Gabriel *et al.*, 2011). Improvements in designed ZFN specificity (either via modification of the nuclease or DNA binding domains) could further reduce the risk of off-target cleavage. In our clinical trial NCT00842634 to date, we have not observed any adverse events attributed to ZFN gene editing in patients.

In the present study, we directly compared Ad5/F35 ZFN gene editing of *CCR5* in CD4+ T cells expanded via our modified clinical scale process of T-cell expansion, which incorporates a dynamic perfusion method (Hami *et al.*, 2004; Hollyman *et al.*, 2009) with our previously described research scale process for T-cell expansion (Levine *et al.*, 1997). The results shown validate that our clinical scale process of T-cell expansion can produce an expanded Ad5/F35 ZFN-CCR5-modified CD4+ T-cell population that shows enhanced growth capacity, cytokine and chemokine production, and comparable phenotype and TCR diversity to previous research scale expansion methods.

Our previous *in vivo* study using a mouse model of acute HIV infection showed, for the first time, that ZFN-guided genomic editing is highly specific, well tolerated, and sufficiently robust to support engraftment and HIV resistance in that model (Perez *et al.*, 2008). In consultation with the FDA, we expanded on these studies to evaluate possible toxicity caused by the CCR5-ZFN. In our toxicity studies, the predominant findings of microscopic histopathology were consistent with xGVHD in all studies. No treatment-related toxicity, including CCR5-ZFN or T-cell-related tumor formation, was observed in any animals. While the NSG model was the best available animal model for evaluation of *in vivo* toxicity and biodistribution of genetically modified human cells, it is not ideal due to xenogenic-mediated pauci-clonal expansion leading to TCR restriction and differences in the trafficking of human cells in mice (Lin *et al.*, 2007). There is importance in using animals to establish the safety of a modified cell product as required by the FDA, but in the future we hope that more informative studies can be developed in concert with the FDA that are less expensive and time consuming while still serving to ameliorate the risk to human patients.

Recent work by Holt *et al.* (2010) has shown that the same ZFN-CCR5 construct used in our preclinical studies shown

here and previously (Perez *et al.*, 2008) can be adapted to an additional transient gene delivery system (nucleofection) to effectively knock out *CCR5* in human hematopoietic stem/progenitor cells (HSPCs). This yielded a cell population that retains functional capacities for both engraftment and hematopoietic reconstitution in the NSG mouse, which has been used for modeling HIV-1 infection *in vivo* (Kumar *et al.*, 2008) and human hematopoiesis (Ishikawa *et al.*, 2005). Consistent with our findings in both the NSG and NOG murine models, Holt *et al.* showed that engrafted human CD34+ HSPCs containing a minority of *CCR5*-deleted cells were capable of producing a human polyclonal T-cell population that survived viral challenge with CCR5-tropic HIV-1.

The potential therapeutic benefit of a *CCR5*-specific genetic knockout could confer long term resistance to HIV-1 infection, at least in the modified cells. Hutter *et al.* (2009) reported an HIV-1+ patient who underwent an allogeneic stem cell transplant for treatment of his acute myeloid leukemia with donor cells homozygous for the *CCR5* 32-bp deletion (Δ32). Following chemotherapy, antiretroviral drugs were discontinued, and the patient remained off antiretroviral drugs following stem cell transplantation without return of viremia. The investigators recently reported reconstitution of CD4+ T cells in peripheral blood, gut, and tissue with donor-derived cells, and that HIV remains undetectable in bone marrow, gut, brain/CSF, and blood of this patient at 45 months post-transplant (Allers *et al.*, 2011). This example provides further proof of concept for provision of *CCR5* gene-edited T cells as a potential treatment for HIV infection.

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Author Disclosure Statement

Sangamo BioSciences provided research funding for these studies. Sangamo has not selected or edited the data presented. The University of Pennsylvania coauthors have no personal financial relationship with Sangamo. B.L. and C.J. have a financial interest due to intellectual property and patents in the field of cell and gene therapy. Conflict of interest is managed in accordance with University of Pennsylvania policy and oversight.

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Address correspondence to:
Dr. Bruce L. Levine
Clinical Cell and Vaccine Production Facility
Hospital of the University of Pennsylvania
Maloney Bldg, Room M6.40
3400 Spruce Street
Philadelphia, PA 19104

E-mail: levinebl@mail.med.upenn.edu

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