Sustained Expression of α_1-Antitrypsin after Transplantation of Manipulated Hematopoietic Stem Cells

Andrew A. Wilson, Letty W. Kwok, Avi-Hai Hovav, Sarah J. Ohle, Frederic F. Little, Alan Fine, and Darrell N. Kotton

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MATERIALS AND METHODS

Generation of dual promoter and IRES-containing lentiviral constructs for dual transgenesis

Lentiviral constructs utilized the third generation, self-inactivating, replication incompetent lentiviral backbone vector originally published as pHR' (21) and modified into the 'pHAGE' vector(22), a generous gift of Dr. Richard C. Mulligan (Harvard Medical School, Boston, MA). The pHAGE vector was modified for dual transgenesis as follows: cDNA encoding a variant of the red fluorescent protein adapted from *Discosoma sp.* (DsRed-Express; Clontech, Mountain View, CA) was amplified by PCR attaching NotI and BamH1 restriction sites to 5' and 3' ends respectively. This amplicon was cloned into the pHAGE backbone in the first gene expression position by ligation to NotI/BamH1 cohesive ends. Next, enhanced green fluorescence protein (GFP; Clontech) cDNA was generated by PCR attaching NdeI and ClaI sites to the 5' and 3' ends respectively for ligation into the second gene position of pHAGE. Immediately upstream of the dsRed or GFP ATG start site, the indicated promoter fragment (cytomegalovirus (CMV), 584 bps; phosphoglycerate kinase (PGK), 464 bps; ubiquitin C (UBC), 397 bps; or elongation factor 1 alpha (EF1α), 228 bps(22)) was inserted by standard cloning techniques as illustrated in Figure 1. For bicistronic vectors, the internal ribosome entry site (IRES) from the encephalomyocarditis virus was inserted (Figure 1; sequences available for download at www.kottonlab.com) immediately upstream of the second cistron's ATG start site.

Generation of AAT-containing lentiviral constructs and viral packaging

Lentiviral constructs for human AAT expression were generated using full-length (1257bp) human AAT (hAAT) cDNA cloned from the c-AT plasmid, a generous gift of Drs. Terry R. Flotte and Sihong Song (University of Florida, Gainesville, FL; Figure 1) (8). PCR was used to add Not I and Bgl II restriction sites upstream and downstream of the 5' hAAT ATG start site and 3' TAA stop site, respectively. This amplicon was ligated into the first gene position of the indicated single or dual transgenesis pHAGE, lentiviral construct (Figure 1) by standard directional cloning into compatible NotI and BamH1 sites.

Vesicular stomatitis virus (VSV) pseudotyped lentivirus was generated by quintuple transfection of 293T cells with the lentiviral backbone construct together with four helper plasmids encoding the viral genes Gag-Pol, Tat, Rev, and VSV-G(20, 21). Cell supernatants containing virus were concentrated by centrifugation (90 minutes; 48960 x g). Titers of fluorochrome-expressing lentiviruses were calculated as '293-transducing units' per ml (TU/ml) based on flow cytometry of infected 293 cells. Titers of viruses lacking a fluorochrome were determined by p24 enzyme-linked immunosorbent assay (ELISA) kit (Cell Biolabs, San Diego, CA) according to the manufacturer's instructions and equivalent TU/ml were calculated.

HSC Isolation, Transduction, and Transplantation

Donor 11 week old B6.SJL-PtprcaPep3b/BoyJ (CD45.1; Jackson Labs, Bar Harbor, ME) mice expressing the Ly5.1 isoform of CD45 were sacrificed and their bone marrow extracted from tibias, femurs, and iliac crests using a mortar and pestle. HSCs were purified from marrow by selection of Hoechst effluxing 'side population' (SP) cells by flow cytometry as published by

our lab and others(23-26). Marrow cells were filtered through a 70-μm Falcon cell strainer to remove debris (BD Biosciences, San Jose, CA), and resuspended at a density of $4.5x10^6$ cells/ml in HBSS+ (calcium- and magnesium-free Hanks balanced salt solution supplemented with 2% heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin, and 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid]; Invitrogen, Carlsbad, CA). Cells were then stained with Hoechst 33342 dye (8.8 μg/ml; Molecular Probes, Eugene, OR) for 90 minutes at 37°C. Stained cells were layered over Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ) before centrifugation to deplete red blood cells (20 minutes at 830 x g). The buffy coat was collected and washed in phosphate buffered saline with 2% fetal bovine serum (PBS+) before resuspension in PBS+ with propidium iodide (PI; 2 μg/ml; Molecular Probes) for cell sorting. HSCs identified as bone marrow SP cells were sorted using a MoFlow triple laser high speed sorting instrument (DakoCytomation, Fort Collins, CO) based on PI exclusion, forward scatter, and Hoechst blue and red parameters as previously published (25). Sorted HSCs were incubated overnight in culture (37C, 5%CO2) with lentivirus at an MOI of 200 under 'minimally stimulating conditions' (20)defined as serum-free Stempro 34 media (Invitrogen) supplemented with 10 ng/ml murine stem cell factor (SCF, R&D Systems, Minneapolis, MN), 100 ng/ml human thrombopoietin (TPO, R&D Systems), and 5μg/ml polybrene (Sigma, St. Louis, MO). Immediately after overnight infection, 3000 HSCs were transplanted without selection of transduced cells into 8 week old C57BL/6J (CD45.2; Jackson Labs) recipient mice by retroorbital intravenous injection. All recipients were prepared for transplant by exposure to 9.5 Gy of ionizing radiation 24 hours prior to HSC injection. All animals received soft food and antibiotic-supplemented water (Sulfamethoxazole 600 mg/Trimethoprim 120 mg per 500 ml; Hi-Tech Pharmacal, Amityville, NY) ad libitum for 2

weeks after transplant. Twenty-four weeks after primary transplant, mice were sacrificed and blood, bone marrow, BAL, and lung tissues were harvested for analysis. Bone marrow was harvested from one mouse in each group for secondary transplantation. Ten million unfractionated whole bone marrow cells from each of these mice were secondarily transplanted into four 9-week old irradiated (9.5 Gy) C57BL/6J recipients. Peripheral blood chimerism and gene expression levels in secondarily transplanted recipients were followed for 7 weeks after transplant. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Boston University.

Measurement of in vitro Gene Expression

In vitro gene expression was measured after lentiviral infection of either FG293 cells or primary murine HSCs in culture at the MOI indicated. Cultured cells were harvested and stained with PI in order to exclude dead cells. PI fluorescence and reporter fluorochrome transgene expression (dsRed, GFP, or ZsGreen) were assessed by flow cytometry (BD FACScan, BD Biosciences). Transduction efficiency was calculated based on the percentage of live (PI excluding) cells expressing the relevant fluorochrome reporter. Cell supernatants were collected after 6 days in culture to measure human AAT protein expression by enzyme-linked immunosorbent assay (ELISA) as detailed below. The presence of hAAT in cell supernatants was further demonstrated by immunoblot as follows: 2.5 mcg of total protein was loaded per sample and proteins were separated by 10% sodium dodecyl sulfate/polyacrilaimde gel electrophoresis (SDS-PAGE). hAAT was detected by Western Blot analysis using a rabbit anti-hAAT primary antibody (RDI, Concord, NH; protocol generously provided by Drs. Yuanqing Lu and Sihong Song). Serial dilutions of cell supernatants were incubated with

human neutrophil elastase (Calbiochem, San Diego, CA) in the presence of methoxysuccinyl-ala-ala-pro-val-paranitroanilide (Sigma) to measure bioactivity. Colorimetric change in the presence of substrate was measured using a 96 well plate reader set at 405 nm (protocol generously provided by Drs. McGarry Houghton and Steven Shapiro, University of Pittsburgh, Pittsburgh, PA).

Measurement of in vivo Gene Expression

At 6 week intervals blood cells and plasma samples were obtained from the retroorbital venous plexus of anesthetized recipient mice using heparinized capillary tubes (Drummond Scientific, Broomall, PA). Following exposure to red blood cell lysis buffer (Sigma), blood samples were stained with phycoerythrin (PE) –conjugated anti-CD45.1 (BD Biosciences #553776) and biotinylated anti-CD45.2 monoclonal antibodies (BD Biosciences #553771) followed by streptavidin-labeled peridinin-chlorophyll-protein complex (Per-CP; BD Biosciences #554064) and 2 μg/ml PI prior to flow cytometry analysis. To obtain lung tissue and bronchoalveolar lavage(BAL) specimens, the thorax was opened and the left mainstem bronchus was ligated. The trachea was cannulated with a 22 ga needle and BAL was performed on the right lung with two consecutive 500 μl aliquots of PBS. BAL fluid (BALF) was separated from BAL cells by centrifugation, and BALF was stored at -80°C prior to analysis of urea and human AAT protein content. Following BAL, the left mainstem bronchus ligature was released and the right ventricle of each animal was flushed with 5 ml of cold PBS. One lung from each animal was then harvested for enzyme digestion with Collagenase A and Dispase II (Roche) as previously published (27)in order to prepare lung single cell suspensions. Cell suspensions

were stained with PE-conjugated anti-CD45 antibody (BD Biosciences) or non-specific isotype control IgG prior to suspension in PBS+ with 2 μg/ml PI for flow cytometry(27).

Flow Cytometry

Flow cytometry analysis was performed using a BD FACScan (BD Biosciences, San Jose, CA) and FlowJo analysis software (Treestar,Ashland, OR). A live cell gate was created by excluding cell fragments (low forward scatter events) and PI bright events. For blood, bone marrow, and BAL samples, chimerism was reported as the percentage of CD45+ cells that expressed the CD45.1 donor surface marker. Where indicated, CD45.1+ cells were selected from a CD45.1 versus CD45.2 plot and displayed as a histogram with GFP or ZsGreen activity on the x axis in order to quantify the percentage of donor-derived cells expressing each reporter gene.

Measurement of human alpha-1 antitrypsin (hAAT) protein levels

hAAT protein expression was measured by dual Ab, sandwich enzyme-linked immunosorbent assay (ELISA; protocol generously provided by Drs. Roberto Calcedo and Joanita Figueredo, University of Pennsylvania, Philadelphia, PA). 96-well microtiter plates (Fisher Scientific, Pittsburgh, PA) were coated with rabbit anti-human alpha-1 antitrypsin antibody (Sigma #A 0409) as capture antibody followed by blocking with 3% bovine serum albumin (BSA; Sigma) in PBS. Standards were generated by serial dilutions of human alpha-1 antitrypsin (Sigma) in PBS with 0.5% BSA and 0.05% Tween 20 (Pierce Biotechnology, Rockford, IL). Samples and standards were plated in duplicate and incubated at 4ºC overnight followed by peroxidase conjugated goat anti-human AAT antibody (EY Laboratories, San Mateo, CA, #PA-2115-1).

hAAT was quantified by optical densitometry after incubation with ABTS peroxidase substrate solution (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland). The lower limit of detection for hAAT was 0.39 ng/ml. Calculation of the hAAT level in the ELF of selected animals was performed using the urea dilution method (Quantichrom Urea Assay, Bioassay Systems, Hayward, CA). Measured urea values in the BALF and blood were used to calculate a dilution factor in order to quantify the original ELF hAAT concentration for each animal (28).

Splenocyte stimulation and intracellular cytokine staining

Splenocytes were harvested from individual mice and red blood cells were lysed with ACK buffer. Cells were then washed with PBS + 2% FBS, counted, and resuspended (4×10^6 cells per tube) in RPMI 1640 medium (Cellgro, Herndon, VA.) supplemented with 10 % FBS, 25 mM HEPES, 2 mM L-glutamine, 20 U of penicillin per ml, 20 μg of streptomycin per ml, 1 mM sodium pyruvate and 0.1 mM nonessential amino acids. For T-cell stimulation, cells were incubated with anti-CD28 (2 μg/ml), anti-CD49d (2 μg/ml) and hAAT whole protein (4 μg/ml). Unstimulated cells were incubated with the above reagents except for the protein. The cells were incubated at 37°C for 5 hr and Golgi Plug (BD Biosciences, 2 μl/ml) was added before an additional 12 hr incubation period. Positive control splenocytes were incubated for 6 hr with PMA (2 μg/ml) and Ionomycin (10 μg/ml) and Golgi Plug. After incubation, samples were washed with PBS + 2% FBS and stained with anti-CD8 and anti-CD4 antibodies for 15 min. Permeabilization was performed with Cytofix/Cytoperm solution (BD Biosciences). Cells were washed with 1× Perm/Wash buffer (BD Biosciences) and then stained with antibodies specific to IFN-γ and IL-2 for 30 min. After an additional washing step with 1× Perm/Wash buffer,

cells were fixed in 2% formaldehyde-PBS. (29, 30) Data was collected on a LSR II instrument and analyzed using FlowJo software. Splenocytes from individual mice were stained with CFSE dye (1μM/ml) for 30 min at 37º C. The cells were then washed and plated in 96-well plates (4×10^6 cells/well). Cells were then stimulated in culture with or without hAAT protein (5μg / ml) for 96 hr at 37º C. The samples were then collected, stained with fluorescence-conjugated monoclonal antibodies against CD8, CD4 and CD19 (BD Bioscience), and run on the LSR II cytometer, followed by analysis with FlowJo software. Proliferation of CD4+ or CD8+ lymphocytes in response to hAAT stimulation was calculated by quantifying any dilution in CFSE staining for each cell population.

Serum analysis

Blood was drawn from mice via RV puncture at the time of sacrifice and sera were stored at −70°C prior to use. Anti-hAAT-specific IgG antibodies were measured by ELISA for each mouse. Ninety-six-well plates (Maxisorp,Nunc) were coated overnight at 4°C with 1 μg of the recombinant hAAT antigen/well in 0.1M sodium carbonate (pH 9.5) solution. Plates were washed twice with PBS-0.05% Tween 20 and blocked with PBS-10% FCS (2 hr at room temperature). Subsequently, mouse serum samples diluted serially in PBS were added to the wells for 2 hr incubation at RT. Serial dilutions of hAAT specific rabbit polyclonal serum were added as positive controls. Plates were then washed 5 times with PBS-0.05% Tween 20, and either HRP-conjugated goat anti-mouse IgG (KPL) or HRP-conjugated goat anti rabbit IgG (BIORAD) as appropriate. After incubation for 1 h at room temperature, plates were washed 7 times and 100 1 μl of Sure Blue reagent (KPL) was added. Reactions were stopped after 30

min by addition of TMB stop solution (KPL). Absorption was read at 405 nm using an ELISA reader (SPECTRA max PLUS).

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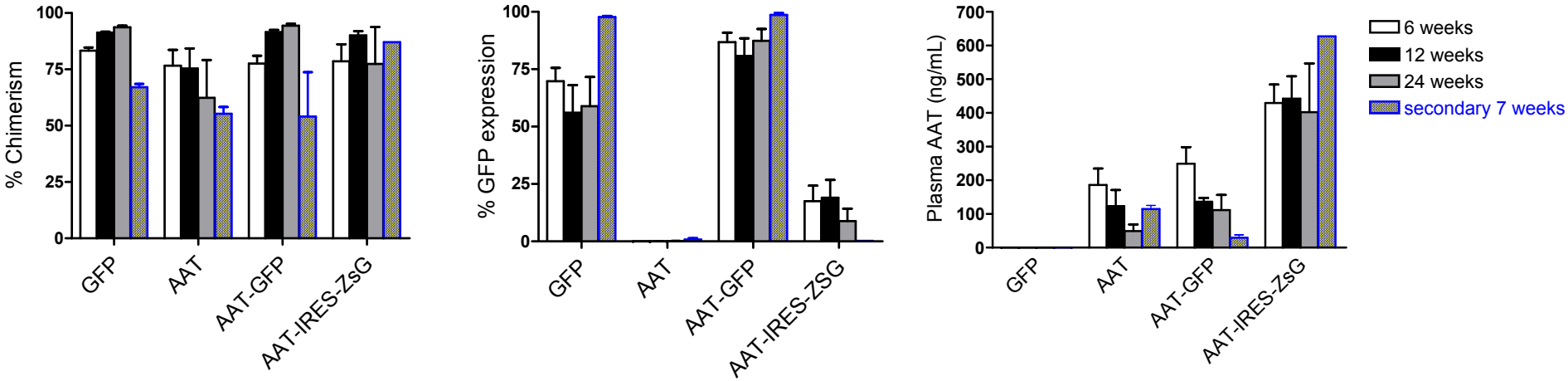
E1.

Figure E1: **Transduced HSCs retain hematopoietic reconstituting capacity after serial transplantation.** 24 weeks after primary HSC transplantation, $10x10^6$ bone marrow cells from one mouse in each group were transplanted into 4 lethally irradiated secondary recipients. After 7 additional weeks, blood chimerism, GFP expression, and plasma AAT expression were measured in each secondary recipient.



E2.

Figure E2: **Absence of Humoral and Cell-Mediated Immune Responses to hAAT Protein Following Transplant of Transduced HSCs.** 18 weeks after primary HSC transplantation, sera and splenocytes were isolated from recipient mice. A: ELISA for anti-hAAT specific antibodies did not reveal a humoral immune response to the human transgene in mice transplanted with HSCs transduced with the dual transgenesis lentivector expressing AAT and GFP (AAT-GFP) when compared to untransplanted control animals. Rabbit anti-hAAT antibodies were added to positive control wells. B: Splenocytes isolated from the same recipients were stimulated with hAAT protein and production of IFN-γ or IL-2 was measured by FACS assay after intracellular cytokine staining (ICS). There was no increase in IFN-γ or IL-2 production by hAAT-stimulated CD4+ or CD8+ T cells from transplant recipients when compared to untransplanted control animals. Splenocytes stimulated with PMA and Ionomycin served as positive controls.