Systemic T Cell–independent Tumor Immunity after Transplantation of Universal Receptor–modified Bone Marrow into SCID Mice

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Summary

Gene modification of hematopoietic stem cells (HSC) with antigen-specific, chimeric, or "universal" immune receptors (URs) is a novel but untested form of targeted immunotherapy. A human immunodeficiency virus (HIV) envelope–specific UR consisting of the extracellular domain of human CD4 linked to the ζ chain of the T cell receptor (CD4ζ) was introduced ex vivo into murine HSC by retroviral transduction. After transplantation into immunodeficient SCID mice, sustained high level expression of CD4ζ was observed in circulating myeloid and natural killer cells. CD4ζ-transplanted mice were protected from challenge with a lethal dose of a disseminated human leukemia expressing HIV envelope. These results demonstrate the ability of chimeric receptors bearing ζ-signaling domains to activate non–T cell effector populations in vivo and thereby mediate systemic immunity.

Introduction of therapeutic genes into hematopoietic stem cells (HSC)[1] may be useful for treatment of human diseases such as HIV infection and cancer. Current gene therapy strategies for AIDS include "intracellular immunization" (1) in which inhibition of HIV infection or propagation in target cells is mediated through the introduction of HIV resistance genes into HSC or mature T cells (2–4), as well as active immunization with gene-modified cells expressing HIV antigens (5, 6). Strategies for cancer gene therapy include active immunization with autologous tumor cells genetically altered ex vivo with immunomodulatory genes, such as costimulatory receptors and cytokines (7, 8). Many of these approaches rely on active as opposed to passive immunotherapy, requiring in vivo stimulation of the host cellular immune system and induction of T cell responses to achieve effective systemic immunity.

In this report, we describe a novel strategy for passive immunotherapy of infectious or malignant disease involving transplantation of HSC modified ex vivo with disease-specific chimeric or "universal" immune receptors. Universal receptors (UR) are HLA-unrestricted chimeric proteins in which the signaling domain of a native immune receptor is fused to a heterologous ligand-binding domain. Cytolytic effectors such as myeloid and NK cells arise rapidly after bone marrow transplantation, and can be specifically directed via URs to diseased cells in vivo. Furthermore, gene-modified HSC provide a continuous source of UR-expressing hematopoietic cells of multiple lineages, which may lead to prolonged systemic immunity.

To date, reports from our laboratory and others have examined in vitro UR function after modification of mature T (9–13) and NK (14) cells. We have previously described two classes of UR in which the signaling domain of the TCR-ζ chain (15, 16) is fused either to a single-chain antibody (SAb) specific for the HIV envelope (HIV-env) glycoprotein, gp41, or to the extracellular domain of human CD4 specific for HIV-env gp120. Both receptors redirect $CD8^+$ T cells (13) and NK cells (14) to kill HIV-infected cells and HIV-env–expressing tumors in vitro. Although the native ζ chain is primarily associated with the TCR, the cytoplasmic tails of the Fc receptor (FcR)-γ chain and TCR-ζ share a conserved 18–amino acid immunoreceptor tyrosine activation motif (17, 18), and both γ and ζ are present as homo- and heterodimers in some classes of FcR (19–21). This structural similarity suggests that ζ-bearing URs may also activate FcR-mediated effector functions of myeloid cells, such as antibody-dependent cellular cytotoxicity (ADCC). Indeed, preliminary work in this laboratory demonstrates cytolytic activity of CD4ζ-expressing neutrophils against HIV-env–expressing target cells in vitro (our

[1]*Abbreviations used in this paper:* ADDC, antibody-dependent cytotoxicity; APC, allophycocyanin-streptavidin; FcR, Fc receptor; HIV-env, HIV envelope; HSC, hematopoietic stem cells; LDC, low density cells; PB, peripheral blood; Raji-env, Raji cells expressing HIV-env; SAb, single-chain antibody.

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unpublished data). To analyze the in vivo function of non–T cell effector populations bearing ζ-based URs, we transplanted immunodeficient SCID mice that lacked mature T and B cells (22, 23) with bone marrow that were retrovirally-transduced with the CD4ζ UR. Transplanted mice were analyzed for UR expression and subsequently challenged with a human B cell leukemia stably expressing HIV-env.

Materials and Methods

Retroviral Vectors. The retroviral vector rkat43.3F3 is a variant of the previously described rkat43.2F3 vector (24) in which an internal human phosphoglycerol kinase (PGK) promoter has been inserted upstream of the CD4ζ coding sequence. Retroviral supernatants were prepared by transient transfection of human 293 cells with rkat43.3F3 and a plasmid containing packaging functions (pkat) using the *kat* system (24).

HIV-specific CD4 and SAb URs. The CD4ζ UR was constructed as described previously (13). The HIVgp120-specific SAbζ UR was constructed as described for the HIVgp41-specific SAbζ UR (13), with the following modification: the extracellular domain of the gp120-SAbζ UR was derived from the gp120-specific human mAb, 447-D (25). The cytoplasmic and transmembrane domains are identical to that of CD4ζ, deriving from TCRζ and CD4, respectively.

Animals. C.B-17 *scid/scid* (SCID) mice were used for all transplant studies. Bone marrow donors were 8–16-wk-old male and female mice obtained from the Cell Genesys in-house SCID colony. Bone marrow recipients were 8–12-wk-old male SCID mice procured from an outside vendor (Charles River Laboratories, Wilmington, MA). Animals were housed in sterile laminar airflow hoods and fed ad lib with sterile food and water. Before use in transplantation experiments, mice were screened for serum IgM levels by ELISA as previously described (26). Only mice with <0.5 μg/ml IgM were used. All animal procedures conformed to institutional guidelines.

Retroviral Transduction and Transplantation of SCID Mice. Donor SCID mice were injected via the tail vein with 5-fluorouracil (100 μg/kg; Roche Laboratories, Hoffmann-La Roche Inc., Nutley, NJ). 6 d later, mice were killed by CO_2 asphyxiation. Femurs were harvested and flushed with culture medium (DME/4.5 g/liter glucose, 15% FCS, glutamine, penicillin, and streptomycin) + 5 mM EDTA. Low density cells (LDC) were isolated by density gradient separation using Lympholyte-M (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada). Briefly, bone marrow cells were layered over an equal volume of gradient and spun at 2,200 rpm for 20 min at 20°C in a tabletop centrifuge. Interphase cells were collected, washed, and resuspended in culture media. LDC were plated at 3×10^6 cells/well of a six-well plate (Corning Glass Inc., Corning, NY) and exposed to UR-expressing retroviral supernatant (CD4ζ or SAbζ) containing 8 μg/ml polybrene on plates coated with rat fibronectin (15 mg/well of six-well plate in PBS; Sigma Immunochemicals, St. Louis, MO; 27). After 2 h, the medium was hemidepleted and fresh viral supernatant was added. Viral supernatant was prepared by transfection of 293 cells using the *kat* system, as described previously (24). Transduced cells were harvested from the plates after 4 h, washed, and resuspended in 0.9% NS + 0.1% BSA for injection. 10^6 transduced LDC/mouse were infused into sublethally irradiated (350 rads) SCID mice via tail vein injection.

Immunofluorescence Analysis of CD4ζ. 300 μl of heparinized blood was attained by retroorbital bleeds of CD4ζ transplanted and control mice at various time points after transplant. RBCs were depleted by ammonium chloride lysis. Approximately 2×10^5 cells/stain were incubated with the following murine-specific mAbs conjugated to FITC: anti–GR-1, anti–Mac-3, anti-5E6 (Pharmingen, San Diego, CA) in addition to anti–human CD4-PE (Becton Dickinson & Co., Mountain View, CA), according to manufacturer's instructions. FITC- and PE-conjugated isotype-matched mAbs served as negative controls. Stained cells were analyzed on a FACScan® cytometer (Becton Dickinson).

Quantitative-Competitive PCR Assay for CD4ζ. 20,000 RBC-depleted peripheral blood cells isolated from CD4ζ-transplanted mice (e^-) were combined with known titrations of transduced competitor cells in which the CD4ζ construct contained an additional 102-bp insert comprised of the 3′ end of the Moloney murine leukemia virus envelope gene (e^+). Cell mixtures were washed, resuspended in 50 μl of lysis buffer (1× PCR buffer (Fisher Scientific, Pittsburgh, PA), 0.1% Tween 20, 100 μg/ml proteinase K (Boehringer Mannheim, Indianapolis, IN) and heated to 55°C for 45 min, followed by 95°C for 15 min. PCR was performed using a primer set that recognizes the integrated CD4-ζ construct (upper, 5′AACTGATTGGTTAGTTCAAAT-AAGGC3′; lower, 5′CCAGACCTGCAGACGCCCAGA3′). 2 μl of cell lysate was added to 48 μl of PCR reaction mix (25 pmol each primer, 1× PCR buffer, 1.5 mM $MgCl_2$, 5 U Taq polymerase [Fisher], 50 μM dNTP). Cycling was initiated in a thermocycler (model 9600; Perkin-Elmer Corp., Norwalk, CT, 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, 26 cycles). The PCR products were electrophoresed through preformed 6% polyacrylamide gels (Novex, San Diego, CA) and resolved by autoradiography.

PCR Assay for Detection of Raji Cells Expressing HIV-env. 300 μl of heparinized peripheral blood (PB) was attained by retroorbital bleeds from eight surviving mice 4 mo after Raji cells expressing HIV-env (Raji-env) challenge and a control mouse. Ammonium chloride RBC lysis was performed and recovered cells were washed, pelleted, and resuspended at 2,000 cell/μl in lysis buffer (1× PCR buffer [Fisher], 0.1% Tween 20, and 100 μg/ml proteinase K [Boehringer Mannheim]). As a positive control, lysates of 0.1, 1, and 10 cultured Raji-env cells in a background of 10^4 NIH 3T3 cells were also prepared. The samples were heated at 55°C for 45 min, followed by 95°C for 15 min to inactivate the proteinase K. PCR was then performed using two sets of primers: (*a*) murine β-actin (upper 5′CGAGCATCCCCCAAAGTTCA-CAA3′; lower 5′CCCAGC-CACACCACAAAGTCACA3′) and (*b*) herv-H (upper 5′ACT-ATAGGCAACTTTCCACC-CTCC3′; lower 5′GCTACTTGG-CTGCCT-CTA-CTCTA3′). Reactions were set up in a 50-μl vol with 5 μl of cell lysate, 1× PCR buffer, 1.5 mM $MgCl_2$, 25 pmol of each primer, 50 μM dNTP, and 5 U Taq polymerase (Fisher). Cycling was initiated in a Perkin-Elmer thermocycler (94°C for 1 min, 60°C for 1 min, 72°C for 2 min, 30 cycles). The PCR products were electrophoresed through preformed 6% polyacrylamide gels (Novex) and resolved by autoradiography.

Recovery of Raji-env from Transplanted Mice. At the time of death from disseminated leukemia, femoral bone marrow was harvested from a CD4ζ-transduced and a control mouse injected with Raji-env. Bone marrow cells were subjected to ammonium chloride RBC lysis and then incubated with saturating concentrations of anti–human CD19-PE (Becton Dickinson) for detection of Raji-env cells. $CD19^+$ Raji-env cells were then sorted using the Becton Dickinson FACStar Plus®. Recovered cells were subjected to flow cytometric and immunoblot analysis to detect the HIV-env protein gp120.

Immunofluorescence Analysis of Raji-env. 10^6 sorted Raji-env cells,

as well as parental Raji cells (Raji-p) and Raji-env cells maintained in liquid culture, were incubated with mouse anti-gp120 mAb (New England Nuclear [NEN] Virus Research, Wilmington, DE) or the isotype-negative control, followed by incubation with goat anti–mouse biotin $F(ab')_2$ (Cappel Laboratories, Durham, NC) and allophycocyanin-streptavidin (APC; Molecular Probes, Inc., Eugene, OR). APC-stained cells were analyzed using a Becton Dickinson FACStar Plus®.

Immunoblot Analysis of Raji-env. 10^6 sorted Raji-env cells and cultured Raji-p and Raji-env cells were lysed for 30 min at 4°C in 10 μl of NP-40 lysis buffer (1% NP-40, 150 mM NaCl, and 10 mM Tris [pH 7.8]). Lysates were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and incubated with anti-gp120 mAb (NEN Virus Research). Bound Ab was detected with horseradish peroxidase–conjugated sheep Ab to mouse IgG, followed by a nonisotopic enhanced chemiluminescence assay (Amersham, Arlington Heights, IL).

Neutrophil Isolation and Cytotoxicity Assay. 3 wk after transplant, four CD4ζ-transduced and four control mice were treated with seven daily subcutaneous injections of human G-CSF (100 μg/kg per d; Amgen, Thousand Oaks, CA). Mice were then killed, and heparinized blood was recovered by cardiac puncture. Neutrophils were isolated using a modification of the standard "1-step Polymorph" procedure (Accurate Chemical & Science Corp., Westbury, NY) (28) in which 0.8 ml of 1.5% NaCl was added to 10 ml of stock gradient. 4 ml of murine PB was layered over 4 ml of modified gradient, and tubes were spun for 30 min at 450 *g* at 20°C in a tabletop centrifuge. The neutrophil fraction was recovered and washed twice. An aliquot was removed for cytospin analysis and Wright-Giemsa staining using standard techniques. Additional cells were removed for measurement of CD4ζ transduction efficiency by flow cytometric and quantitative-competitive (QC)-PCR analysis. The remaining cells were used in a chromium release cytotoxicity assay. Raji-p and Raji-env cells were labeled with sodium [^{51}Cr] chromate (100 μCi/10^6 cells) for 3 h at 37°C, washed three times, and resuspended in assay medium (RPMI, 10% FCS, 10 ng/ml murine GM-CSF). 10^5 ^{51}Cr-labeled target cells were plated in duplicate in 96-well plates together with control or CD4ζ-expressing neutrophils at E/T ratios of 250:1–1.2:1. To measure ADCC, polyclonal rabbit anti–human lymphocyte serum (4 mg/ml; Accurate Chemical & Science Corp.) was added to one row of duplicate wells. Cells were incubated at 37°C for 4 h. 100 μl of supernatant was removed from each well and counted in a gamma counter for the assessment of ^{51}Cr release. The percentage of specific lysis was calculated from duplicate samples using the following formula: $(CMP - SR)/(MR - SR) \times 100$, where *CMP* is the cpm released by targets incubated with effector cells, *MR* is the cpm released by targets lysed with 100 μl of 1% Triton X-100, and *SR* is the cpm released by targets incubated with medium only.

Results

Transplantation of SCID Mice with CD4ζ-modified Bone Marrow. The UR CD4ζ was introduced into bone marrow progenitor cells of SCID mice using retroviral transduction. A high efficiency retroviral transduction system, *kat*, was used to generate high titer retroviral supernatants containing the CD4ζ construct from 293 cells transiently transfected with packaging (pkat) and retroviral vector (rkat) plasmids, as described previously (24). Retroviral titers on NIH 3T3 cells ranged from 6×10^6–10^7 viral particles/ml. The retroviral vector used, rkat43.3F3, is Moloney murine leukemia virus based and contains an internal phosphoglycerol kinase (PGK) promoter to minimize loss of transcriptional activity in vivo (29). After reverse transcription and integration into target cells, transcription is initiated only from the PGK promotor. Previous work in this laboratory has shown that this vector yields stable levels of CD4ζ expression over 6 mo in transplanted C3H mice, whereas viral long terminal repeat (LTR) driven expression diminishes rapidly over 1–2 mo (our unpublished data).

Donor SCID mice were treated with 5-fluorouracil to enrich for immature hematopoietic progenitors with long-term repopulating ability (30). LDC were isolated and exposed to CD4ζ retroviral supernatant in the presence of rat fibronectin, which has been shown to increase the efficiency of retroviral gene transfer (27). Transduced cells were then infused via tail vein injection into sublethally irradiated recipient SCID mice.

Sustained, Multilineage Expression of CD4ζ in Transplanted Mice. Long-term multilineage in vivo expression of the CD4ζ UR was achieved after gene transfer into SCID bone marrow. 3 wk after transplant, mice were analyzed for transduction efficiency and CD4ζ expression by QC-PCR and flow cytometric analysis of peripheral blood and bone marrow. In five separate experiments using 20–40 mice each, the mean percentage of PB leukocytes expressing CD4ζ as measured by immunofluorescence was 39% (range = 12–70%). Lineage-specific expression of CD4ζ was measured by double staining with PE-conjugated anti–human CD4 and FITC-conjugated mAbs specific for various murine blood lineages (Fig. 1 *a*). These included Mac-3 (found on monocytes, macrophages, and granulocytes; 31), Gr-1 (expressed on granulocytes; 32), and 5E6 (present on a subset of NK cells; 33). CD4ζ expression was highest in Mac-3^+ monocytes and granulocytes, followed by Gr-1 bright mature granulocytes, and $5E6^+$ NK cells. The absence of circulating $CD4^+$ and $CD8^+$ T cells in these transplanted SCID mice was confirmed by flow cytometry (data not shown). The percentage of bone marrow cells expressing CD4ζ averaged 20–40% of that seen in the PB, suggesting that expression of CD4ζ in hematopoietic cells may be affected by their state of differentiation.

Subsequent QC-PCR analysis of PB from five transplanted mice demonstrated levels of integrated provirus that approximated expression levels determined by immunofluorescence. A representative result is shown in Fig. 1, *b* and *c*. To confirm that our transduction system targeted long-term repopulating HSC (34), CD4ζ expression in the peripheral blood of transplanted mice was monitored during a period of 6 mo. In a cohort of eight mice followed longitudinally, the mean percentage of peripheral blood leukocytes expressing CD4ζ, as measured by immunofluorescence, was 16% (range = 13–19%) at 3 wk, 14% (range = 6–23%) at 4 mo, and 17% (range = 5–28%) at 6 mo.

CD4ζ-expressing Mice Demonstrate Immunity to Raji-env. We next tested the in vivo function of hematopoietic cells expressing CD4ζ by challenging transplanted mice with a derivative of the human B cell leukemia/lymphoma, Raji,

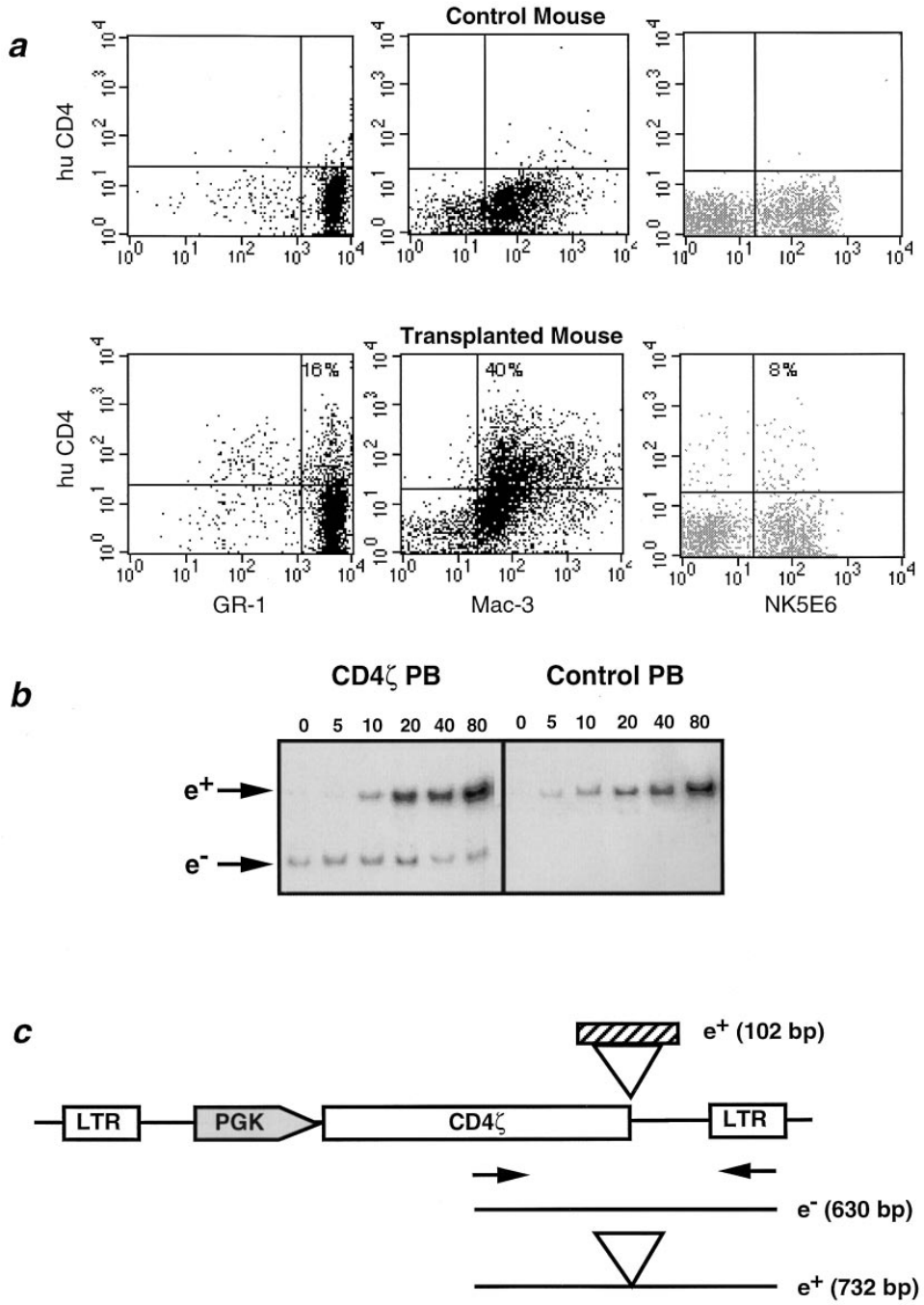

Figure 1. Analysis of CD4ζ expression in transplanted mice. (*a*) Flow cytometric analysis. PB leukocytes from mice 3 wk after transplant were incubated with the following murine-specific mAbs conjugated to FITC: anti–GR-1, anti–Mac-3, anti-5E6 in addition to anti–human CD4-PE. Background human CD4 expression in various lineages in a control mouse (*top*) is compared to human CD4 expression in a CD4ζ transplanted mouse (*bottom*). Specific cell staining was measured on gated populations containing myeloid cells (Gr-1 and Mac-3) and lymphoid cells (NK5E6), as determined by forward and side scatter characteristics. Results are representative of those observed in 80 additional mice. (*b*) QC-PCR analysis. CD4ζ-expressing PB cells from a mouse 3 wk after transplant (e^-) were combined with known titrations of CD4ζ-transduced competitor cells (e^+). The lane in which the e^- and e^+ amplification products are equivalent represents the percentage of PB cells containing the CD4ζ gene; in this case, ~10%. The corresponding expression level by FACS® analysis in this mouse was 20%. PB from an untransplanted mouse serves as a control. (*c*) The structure of the integrated CD4ζ retroviral vector and the competing template used in the QC-PCR assay. The CD4ζ construct used to generate the e^+ competitor cells contains an additional 102-bp sequence to enable differential separation of the PCR products on gel electrophoresis. The location of the PCR primers (*black arrows*) and the expected competing transcripts (e^- and e^+) are shown. *PGK*, human phosphoglycerate kinase promoter.

which stably expresses low levels of the HIV-env proteins gp120 and gp41 (Raji-env; 14). Since HIV gp120 binds to human CD4, Raji-env is a specific target for the CD4ζ UR. Intravenous infusion of Raji-p into SCID mice reproducibly causes a lethal disseminated leukemia that invades the bone marrow, liver, spleen, and central nervous system (26). Preliminary studies were performed to determine the optimal tumor dose for subsequent challenge experiments. Deaths after intravenous infusion of various doses of either Raji-p or Raji-env were as follows: 10^7 (17–22 d), 10^6 (22–25 d), 10^5 (30–60 d), 10^4 (no deaths).

In the first experiment, SCID mice were transplanted with CD4ζ-transduced bone marrow, and 3 wk later were challenged with 10^5 or 10^6 Raji-p or Raji-env cells via tail vein injection. Transplanted mice were then followed for the development of hind leg paralysis secondary to central nervous system invasion and death from disseminated leukemia (Fig. 2 *a*). In the group receiving 10^5 Raji-env cells, 8/10 CD4ζ-expressing mice survived >4 mo after transplant, whereas only 1/10 of the transplanted mice receiving 10^5 Raji-p cells survived (Fig. 2 *a*). All untransplanted mice challenged with either Raji-p or Raji-env died within 60 d.

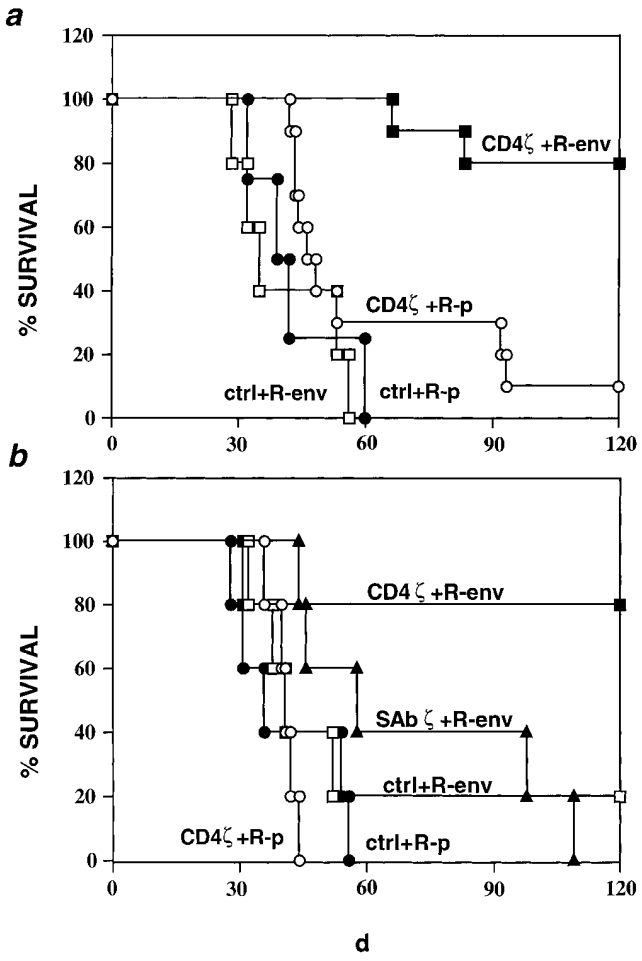

Figure 2. Survival of transplanted mice after Raji-env cell infusion. In two separate experiments, SCID mice were transplanted with UR-transduced bone marrow (CD4ζ or SAbζ). 3 wk after transplant, UR-transduced and control mice were injected via the tail vein with Raji-p or Raji-env tumor cells. (*a*) Survival of CD4ζ mice (10/group) receiving 10^5 Raji-env (*CD4ζ+R-env*) or Raji-p (*CD4ζ+R-p*) cells compared to historical control untransplanted mice (5/group) receiving 10^5 Raji-env (*ctrl+R-env*) or Raji-p (*ctrl+R-p*) cells. Between 4 and 8 mo after transplant, four of the Raji-env survivors died from the spontaneous development of endogenous thymic lymphomas, which is a known complication of sublethal irradiation in SCID mice (51). (*b*) Survival of CD4ζ and SAbζ mice (5/group) challenged with 10^5 Raji-env or Raji-p cells compared with concurrent control untransplanted mice infused with either tumor (5/group). SAbζ mice infused with 10^5 Raji-p all died within 50 d.

At the higher leukemia cell dose of 10^6, CD4ζ-expressing mice receiving Raji-env exhibited a 10–40-d delay in death as compared to Raji-p infused controls, but 9/10 mice died within 80 d (data not shown).

Similar results were observed in a second experiment designed to test the limits of this model by comparing the anti tumor activity mediated by a high versus a low expressing UR. Murine bone marrow was transduced with either the CD4ζ UR or with a SAbζ UR specific for HIV-env gp120 which, in contrast to previous SAbζ URs tested (13), is poorly expressed upon gene transfer. CD4ζ-transduced mice showed surface human CD4 expression in 29–42% of circulating leukocytes at 3 wk after transplant, whereas mice transplanted with the SAbζ gene expressed the UR in only 1–3% of peripheral blood cells by flow cytometry (data not shown). CD4ζ, SAbζ, and untransplanted control mice were challenged with 10^5 Raji-p or Raji-env cells (five per group, Fig. 2 *b*). 4/5 CD4ζ-expressing mice that received Raji-env cells survived >4 mo after infusion. In contrast, all five SAbζ-transplanted mice succumbed to the disseminated Raji-env leukemia, as did 9/10 control untransplanted mice challenged with either Raji-p or Raji-env, and all CD4ζ- and SAbζ-transplanted mice receiving Raji-p. This study confirms the in vivo antitumor activity of

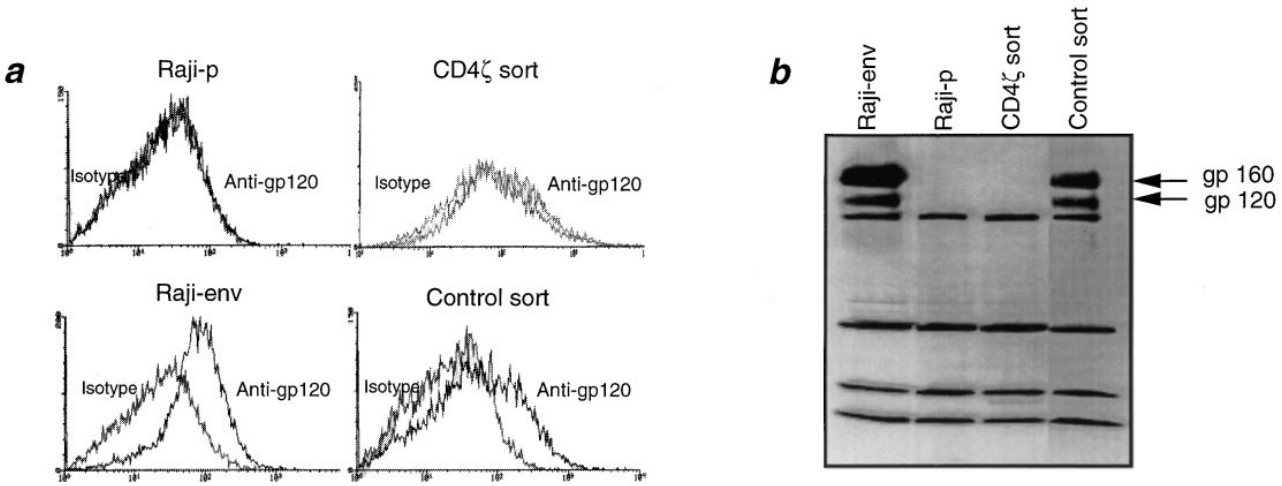

Figure 3. Death of CD4ζ-transplanted mice after Raji-env infusion is associated with a loss of gp120 expression in vivo. (*a*) Flow cytometric analysis. Raji-env cells sorted from the bone marrow of a CD4ζ-transplanted (*CD4ζ sort*) and a control mouse (*control sort*), as well as Raji-p and Raji-env cells maintained in liquid culture, were incubated with mouse anti-gp120 mAb to detect surface expression of HIV-env or the isotype-negative control, followed by incubation with goat anti–mouse biotin $F(ab')_2$ and APC (Molecular Probes). (*b*) Immunoblot analysis. Sorted Raji-env cells (*CD4ζ sort* and *control sort*) and cultured Raji-p and Raji-env cells were lysed and subjected to SDS-PAGE, followed by immunoblotting with anti-gp120 mAb to detect the presence of the env protein.

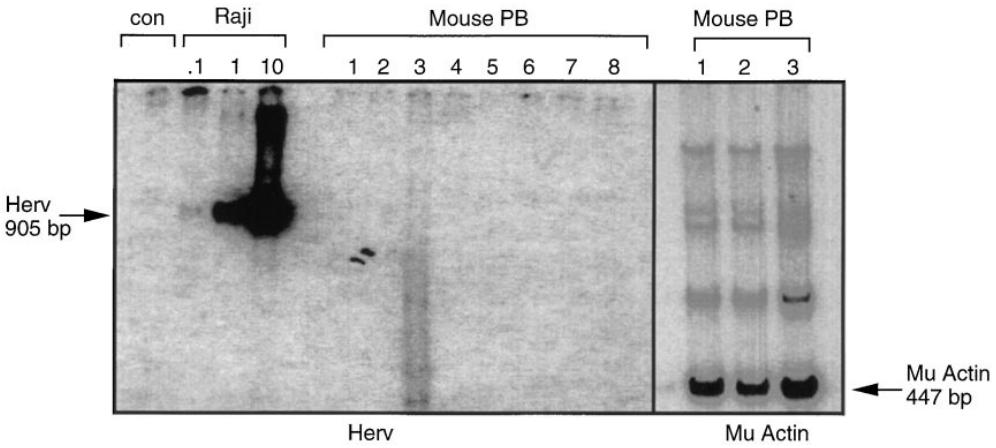

Figure 4. PCR analysis of surviving mice shows no evidence of residual Raji-env. Lysates of PB were prepared from eight surviving CD4ζ-transduced mice challenged with Raji-env at 4 mo after transplant. DNA was amplified using primers specific for a 905-bp human endogenous retroviral sequence (Herv-H) to detect Raji-env DNA (*Mouse PB*, lanes *1–8*). Results are compared to titrations of cultured Raji-env cells in a background of 10^4 murine cells amplified with Herv-H primers (*Raji*) and Herv-H–amplified PB from a control mouse (*con*). Samples were amplified with murine β-actin primers (447-bp product) as a positive control (*Mouse PB*, lanes *1–3*).

CD4ζ-bearing hematopoietic cells seen in the first experiment and shows a relative lack of SAbζ UR–mediated activity, which is likely secondary to low in vivo expression levels. Furthermore, this study rules out the possibility that survival of the CD4ζ mice challenged with Raji-env is caused by a nonspecific effect of the radiation or transplant regimen.

Outgrowth of Env⁻ Raji Revertants in Vivo. In the first experiment, it was noted that death was delayed in the two CD4ζ-expressing mice that died after challenge with 10^5 Raji-env cells (Fig. 2 *a*). To assay for the maintenance of gp120 expression by Raji-env in vivo, bone marrow was harvested from one of these mice at the time of death, and Raji-env cells were isolated by cell sorting using the human anti-B cell mAb anti–Leu-12 (CD19). Human $CD19^+$ Raji cells constituted 4% of bone marrow leukocytes at the time of death from disseminated leukemia in this mouse. Recovered Raji-env cells were subjected to a sensitive APC staining procedure to detect surface expression of HIV gp120 (Fig. 3 *a*), as well as immunoblot analysis to detect total protein (Fig. 3 *b*). HIV gp120 could not be detected in these cells by either technique, suggesting that delayed death of this animal resulted from the outgrowth of Raji-env^- revertants. In contrast, Raji-env cells sorted from the bone marrow of a control mouse at the time of death maintained stable expression of HIV gp120 by both flow cytometric and immunoblot analyses (Fig. 3, *a* and *b*). In subsequent experiments, death after infusion of 10^5 Raji-env cells into CD4ζ-transplanted mice was associated with a loss of gp120 expression in three out of three mice analyzed.

Absence of Detectable Raji-env in Surviving Mice. To screen for residual circulating leukemia cells in surviving CD4ζ-expressing mice challenged with Raji-env, PCR analysis was carried out on PB 4 mo after transplant (Fig. 4). The PCR assay used a probe specific for a human endogenous retroviral sequence, Herv-H, which is present in multiple copies in all human cells (35, 36). The sensitivity of this assay is sufficient to detect a single Raji cell in a background of 10^5 murine cells. All surviving mice were PCR negative, ruling out the presence of minimal residual disease at this level of detection.

In Vitro Neutrophil Cytotoxicity. Previous studies from our laboratory have shown that ζ-bearing URs can direct the cytolytic activity of FcR-bearing nonlymphoid effector cells such as NK cells in vitro (14). Since 70–80% of circulating leukocytes in the SCID mouse are neutrophils (23), we sought to determine whether neutrophils harvested from UR-transplanted SCID mice could demonstrate tumor-specific cytolytic activity in vitro. In three separate experiments, neutrophils isolated from the peripheral blood of CD4ζ-expressing mice demonstrated low level but specific cytolysis of Raji-env targets in a chromium release cytotoxicity assay. One representative experiment is shown in Fig. 5. After correction of the E/T ratio for the percentage of CD4ζ-expressing cells in the bulk neutrophil population (i.e., 8%), UR-mediated target cell lysis approached FcR-mediated ADCC. Because of limitations in the absolute number of CD4ζ-expressing neutrophils that could be isolated from these mice, and the observation that neutrophil ADCC is optimal at E/T ratios in excess of 100:1 (37–39), E/T ratios necessary for maximal killing were probably not reached in these assays. Nevertheless, these in vitro data suggest a potential role for CD4ζ-expressing neutrophils in mediating the antitumor effect observed in vivo. We are currently using the SCID transplant model to investigate the relative contributions of specific UR-expressing lineages such as NK cells, monocytes, and neutrophils in mediating protective tumor immunity in vivo.

Discussion

We have shown that murine SCID long-term repopulating HSC can be efficiently transduced with the UR CD4ζ using a retroviral gene transfer system. Multilineage expression of CD4ζ was documented at high levels in myeloid

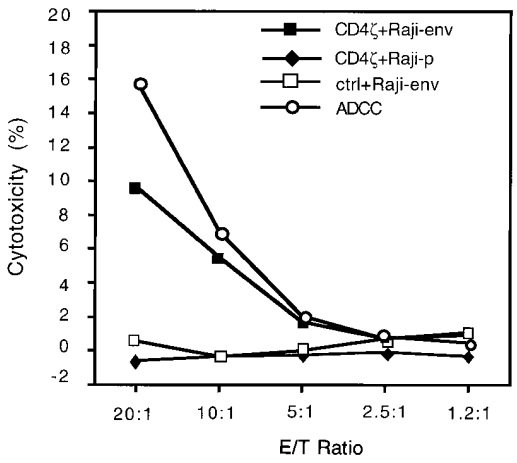

Figure 5. CD4ζ-expressing neutrophils lyse Raji-env targets in vitro. Cytotoxicity assays were performed using neutrophils isolated from CD4ζ-transplanted or control mice as effectors and Raji-p or Raji-env cells as targets. E/T ratios were corrected for the percentage of the bulk neutrophil population expressing CD4ζ by FACS® analysis. FcR-mediated ADCC was measured by incubation of neutrophils with Raji-p in the presence of polyclonal anti–human lymphocyte serum. Maximal cytolysis of Raji-env cells by CD4ζ-expressing neutrophils in three experiments was 10%, 9%, and 8% at corrected E/T ratios of 20:1, 30:1, and 25:1, respectively. Background cytolysis by controls (*CD4ζ neutrophils+Raji-p* and untransduced (*ctrl*) neutrophils+*Raji-env*) was always <1%, even at E/T ratios as high as 400:1.

and NK cells present in PB and bone marrow, and expression remained stable over 4–6 mo in vivo. 80% of CD4ζ-transplanted mice survived after challenge with a lethal dose of a disseminated HIV-env–expressing human leukemia, and surviving animals showed no detectable evidence of residual circulating leukemia cells by PCR analysis. The data presented here demonstrate that CD4ζ-expressing effector cells are the primary mediators of the antitumor activity observed, a conclusion supported by the following observations: (*a*) Infusion of Raji-p leads to rapid leukemia dissemination and death of CD4ζ-expressing mice; (*b*) Raji-env cells injected into untransplanted control mice are lethal; (*c*) mice transplanted with a poorly expressed UR (SAbζ) succumb after Raji-env challenge; and (*d*) failure of CD4ζ-expressing mice to survive after challenge with Raji-env cells is associated with the outgrowth of env$^-$ Raji revertants in vivo.

These studies show that a UR that bears the signaling domain of ζ can effectively redirect effector cells of non–T cell lineages both in vivo and in vitro. In contrast to T cells, FcRs function as the primary immune receptors for myeloid and NK cells. Like the TCR-ζ chain, the γ chain of the FcR mediates intracellular signal transduction (21). FcγRIIIA (found on macrophages and NK cells) and FcεRI (found on eosinophils, basophils, and mast cells) are multiunit protein complexes containing cytoplasmic γ–γ homodimers or γ–ζ heterodimers that function as connectors to signal transduction (18–20). FcγRI (present on monocyte/macrophages and activated neutrophils) physically associates with the homodimeric γ subunit of FcεRI, which may act as a signaling intermediate (40, 41). The genes for the FcR-γ and TCR-ζ chains belong to the same gene family and share a 34% sequence homology at the amino acid level (17). The cytoplasmic tails of γ and ζ share a conserved 18–amino acid immunoreceptor tyrosine motif that becomes phosphorylated in response to immune receptor cross-linking by associated tyrosine protein kinases. Subsequent activation of signal transduction pathways results in effector functions such as cytolysis, phagocytosis, and mediator release (18, 42). Although ζ is functionally associated with γ in a subset of FcRs, and structural similarity exists between these two chains, URs containing the cytoplasmic domain of γ rather than ζ may exhibit enhanced or differential function in myeloid and NK cells. It will, therefore, be of interest in future studies to compare the relative activity of ζ- and γ-based URs in this SCID model.

Many groups, including our own, have focused on T cell adoptive immunotherapy approaches to the treatment of viral infections and cancer. The data presented here, using a SCID model in which functional lymphocytes are absent, suggest a possible role for the manipulation of myeloid and NK cells in the treatment of such diseases. It has been shown that monocytes from HIV$^+$ patients demonstrate significant ADCC against HIV-infected T cell targets, and that neutrophil and mononuclear cell ADCC is impaired in HIV$^+$ children and adults, suggesting that these effector cells may be important in the setting of HIV infection (43, 44). Furthermore, neutrophils (37–39), macrophages (45–47), and NK cells (48) have been shown to mediate efficient ADCC of tumor cell targets in vitro. FcR-mediated cytotoxicity can be enhanced by exposure of effector cells to various hematopoietic cytokines; for example, neutrophil antitumor ADCC is upregulated by exposure to G-CSF, GM-CSF, and IFN-γ (37–39), and macrophage antitumor ADCC is enhanced by M-CSF, GM-CSF, and IL-3 (45–47). In the SCID transplant model, the CD4ζ UR is presumably functioning via the FcR-signaling pathway to activate cytotoxic mechanisms of myeloid and/or NK cells in vivo. It will be of interest to determine whether UR-mediated effector function may be enhanced by in vivo exposure of transplanted mice to cytokines such as G-CSF or GM-CSF. In this model, leukemia cells are injected intravascularly into the same compartment as the gene-modified effector cells, thereby providing optimal exposure of effectors to tumor. One of the next challenges will be the development of new animal models that permit evaluation of the UR stem cell gene therapy approach in the treatment of established solid tumors.

Introduction of disease-specific chimeric immune receptors into bone marrow progenitor cells is a novel application of HSC gene therapy which, to date, has focused on gene-marking studies, introduction of chemotherapy and HIV-1 resistance genes, and correction of rare single-gene defects (49, 50). This is the first study demonstrating the efficacy of such an HSC-based immunotherapy approach in an in vivo model. Gene modification of HSC may be preferable to modification of terminally differentiated effector cells, such as T or NK cells, for several reasons: (*a*) multiple effector cells can be simultaneously redirected using a stem

cell approach; (*b*) prolonged in vitro expansion of gene-modified cells, which may negatively impact their in vivo trafficking or function, can be avoided; and (*c*) a renewable source of gene-modified effector cells capable of prolonged antigen-specific immune surveillance may be created. Although the UR model system we have used is targeted to the HIV protein gp120, chimeric immune receptors may be tailored to recognize a variety of viral or tumor-associated antigen targets. This stem cell gene therapy approach could potentially have broad applicability to the field of targeted immunotherapy of malignant disease.

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