1083-8791/06/1212-0001$32.00/0
doi:10.1016/j.bbmt.2006.09.001

Interleukin-7–Engineered Mesenchymal Cells: In Vitro Effects on Naive T-Cell Population

Paolo Sportoletti,[1] Beatrice Del Papa,[1] Mariangela De Ioanni,[1] Lorenzo Moretti,[1] Elisabetta Bonifacio,[1] Vania Lanterna,[1] Alain Bell,[1] Katia Fettucciari,[2] Eugenia Carnevali,[3] Tiziana Zei,[1] Franca Falzetti,[1] Massimo F. Martelli,[1] Antonio Tabilio,[1] Mauro Di Ianni[1]

[1]Hematology and Clinical Immunology Section and [2]General Pathology and Immunology Section, Department of Clinical and Experimental Medicine, Perugia University, Perugia, Italy; [3]Forensic Medicine Institute, Perugia University, Terni, Italy.

Correspondence and reprint requests: Dr. Mauro Di Ianni, Hematology and Clinical Immunology Section, Department of Clinical and Experimental Medicine, Policlinico Monteluce, via Brunamonti 06122, Perugia, Italy (e-mail: mdiianni@libero.it)

Received April 19, 2006; accepted September 2, 2006.

ABSTRACT

T-cell homeostasis is regulated by several molecules; among these, interleukin (IL)-7 plays an essential role in the survival and homeostatic proliferation of peripheral naive T cells. In a previous study, we investigated whether human mesenchymal stromal cells (MSCs) could be engineered with the IL-7 gene to produce functional level of this cytokine. In the present study, we analyzed the impact of different quantities of IL-7 produced by MSCs on the survival and proliferation of a negative immunoselected naive ($CD3^+/CD45RA^+$) T-cell population. Co-cultivation of peripheral naive T cells with MSCs producing low (16 pg/mL) or high (1000 pg/mL) IL-7 levels or in the presence of exogenous IL-7 (0.01 ng/mL and 100 ng/mL) maintained the $CD3^+/CD45RA^+$ naive T-cell phenotype. Chemokine receptor $CCR7^+$ expression was also maintained among this T-cell population. Naive T-cell molecular characteristics were maintained as assessed by the Vβ spectratyping complexity score, which showed the maintenance of a broad T-cell repertoire. No Th1 or Th2 differentiation was observed, as assessed by interferon-γ or IL-4 accumulation. In contrast, only MSCs producing high amounts of IL-7 caused increased activation (CD25 31.2% ± 12% vs 10% ± 3.5%; $P < .05$), proliferation (CD71 17.8±7% vs 9.3%±3, $P < .05$), apoptosis (assessed by annexin V: 18.6% ± 5% vs 14.9% ± 2.6%; $P > .05$), and the phase S cell cycle (15% vs 6.9%, $P > .05$). Exogenous IL-7 exhibited no significant effect. In conclusion, we demonstrated that IL-7 produced by MSCs has a dose-independent effect on naive T-cell survival while exerting a dose-dependent effect on activation/proliferation. Due to the continuous production of IL-7 by engineered cells, our system is more efficacious than exogenous IL-7.

KEY WORDS

Interleukin-7 • Mesenchymal stromal cell • $CD45RA^+$ • Naive T cell

INTRODUCTION

The common leucocyte antigen (CD45) has 2 isoforms, 1 of which, CD45RA, is expressed on naive T cells (ie, T cells that have not yet encountered antigens), and the other, CD45RO, is expressed on memory T cells ($CD45RA^-CD45RO^+$) [1]. Interactions with dendritic cells activate naive T cells into effector and memory T cells, generating primary immune responses. Both naive and memory T-cell populations are highly dependent on interleukin (IL)-7, a pleiotrophic cytokine secreted by stromal cells in the bone marrow and thymus [2-6]. IL-7 appears to keep naive T cells alive and maintain the naive phenotype $CD3^+CD45RA^+$ without the acquisition of $CD3^+CD45RO^+$ phenotype expression, by delivering signals that inhibit apoptosis [7]. Expression of BCL-2 and other mediators of cell survival is enhanced by engagement of IL-7 through its receptor (IL-7R), which is composed of an α chain (CD127) and the common cytokine γ chain (CD132) [8,9]. Naive and memory T-cell responses to IL-7 are controlled by

regulation of IL-7R expression, T-cell receptor (TCR) triggering, and, obviously, the availability of IL-7 [10,11].

Besides supporting survival of naive T cells in the resting state, IL-7 is also required for homeostatic proliferation of peripheral T cells in response to severe T-cell depletion [7,12,13]. Indeed, in humans, T-cell depletion due to disease or related therapies, such as hematopoietic stem cell transplantation, is associated with a rise in circulating IL-7 levels [14]. Despite this, therapy with IL-7 in T-cell–depleted hosts is controversial in terms of triggering autoimmunity and graft-versus-host disease [15-17], as well as enhancing human immunodeficiency virus replication [18].

In the allogeneic transplantation setting, T and B lymphocytes may be deficient in number and function for up to 1 year posttransplantation, and recovery of naive $CD4^+CD45RA^+$ T cells is particularly slow [19]. IL-7 is believed to reconstitute posttransplantation immune competence by accelerating thymic T-cell development and expanding the peripheral pool of naive T cells [20-24]. Consequently, IL-7–engineered cells could be an ideal vehicle for hastening immunologic reconstitution after allogeneic bone marrow transplantation.

With the development of gene delivery systems that allow genetic engineering of cells to express high levels of a specific cytokine, we hypothesized that continuous production of IL-7 by engineered cells would be more efficacious than exogenous cytokine administration. Mesenchymal stromal cells (MSCs) appear to be an ideal vehicle for this. MSCs can be expanded in vitro from a single bone marrow aspiration to produce millions of cells [25] and are easily transduced with retroviral vectors to produce several molecules for long periods [26]. Bone marrow stromal cells (BMSCs), the progenitors of multiple mesenchymal lineages, constitutively produce growth factors and cytokines [27]. On interacting with their ligands, such as fibronectin, collagens, glycosaminoglycans, and proteoglycans (ie, the bone marrow extracellular matrix) [28], these cytokines regulate pluripotent stem cell proliferation and differentiation and serve to mediate their homing and to transmit regulatory signals [29]. Indeed, in vitro studies have shown that BMSC cultures provide required adhesion sites for immature thymocytes; continued feeding of these cultures results in steady production of replicating immature T cells [30].

We previously demonstrated that MSCs can be transduced to produce IL-7 and that the naive $CD45RA^+CCR7^+$ phenotype remains unchanged, with cells protected from apoptosis and low-profile proliferation maintained [31]. In the present study, we have developed engineered MSCs to produce different amounts of IL-7 for studying the effects of different dosages on survival and proliferation of a $CD45RA^+$-enriched peripheral T-cell population.

MATERIALS AND METHODS

Retroviral Vector and Packaging Cell Line

Construction of the LXSN-NeoR/IL-7 and pBABE-NeoR/IL-7 retroviral vectors started from the Moloney murine leukemia virus (MMLV) [32]. The vector contains the IL-7 gene under the control of a promoter within the LTR sequence and the neomycin-resistance gene (NeoR) under the control of the SV-40 virus. The Pstl-KpnI fragment containing the IL-7 cDNA (500 pb) was blunted and then cloned in the HpaI cleaving site of the LXSN retroviral vector and in the SnabI cleaving site of the pBABE retroviral vector.

The GP+envAM12/LXSN-NeoR/IL-7 and GP+envAM12/pBABE-NeoR/IL-7 vector-producing cell lines were obtained using the ping-pong amplification procedure [33] and had a titer of 1×10^6 retroviral particles/mL.

Human MSC Cultures and Transduction

MSCs were obtained from bone marrow aspirate from healthy subjects who had given informed consent, as described elsewhere [34]. After centrifugation on a Ficoll-Hypaque density gradient (1.077 g/mL), marrow mononuclear cells were resuspended at a concentration of 2×10^6 cells/mL in complete culture medium. Medium contained alpha-MEM (GIBCO BRL Life Technologies, Grand Island, NY), 10% human serum, L-glutamine (2 mmol/L), 2-mercaptophenol (10^{-4} mol/L), inositol (0.2 mmol/L), folic acid (20 μmol/L), and hydrocortisone (10^{-6}mol/L). Cells were cultured in 175-cm^2 flasks and incubated at 37°C 5% CO_2 in a humidified thermostat for 2-4 weeks until the confluent layer was formed. Then, 24 hours after the packaging cells (6×10^6) were seeded on 175-cm^2 flasks, the medium was recovered and filtered through 0.45-μm pore filters. After 15 days of long-term culture, MSCs were infected at a concentration of 3×10^4/mL by adding the viral supernatant in a ratio of 1:1 to the culture medium in the presence of polybrene (8 μg/mL). The cell suspension was kept for 24 hours at 37°C, 5% CO_2 in a humidified thermostat. After 2 infection cycles, cells were selected for 10 days in G418 (0.6 mg/mL). MSCs were detached using trypsin EDTA, washed, resuspended in phosphate-buffered saline, supplemented with human serum fetal bovine serum (1%), and incubated at a concentration of 1×10^6/mL for 30 min at 4°C with fluorescein isothiocyanate (FITC)-conjugated or phycoerythrin (PE)-conjugated monoclonal antibodies, including CD45-FITC, CD14-FITC, CD90-FITC, and CD105-FITC (Beckman Coulter, Hialeah, FL)

and STRO-I-PE (Caltag Laboratories, Burlingame, CA). Each fluorescence analysis included appropriate FITC- and PE-conjugated negative isotype controls. Cells were analyzed using an Epics XL cytometer (Beckman Coulter).

Molecular Analysis of Transduced Populations

The presence of the NeoR gene was confirmed by polymerase chain reaction (PCR) in producing cells and the engineered MSCs. A sample of 1×10^6 cells was lysed in 500 μL of buffer containing Tris HCl 10 mmol, KCl 50 mmol, $MgCl_2$ 2.5 mmol, gelatin 0.1%, NP40 0.45%, Tween 20 0.45%, and proteinase K 100 mg/mL for 60 minutes at 56°C, followed by 10 minutes at 95°C. A 10-μL sample of lysate was used in the PCR reaction with the following NeoR-specific primers: 5′-GGT GGA GAG GCT ATT CGG CTA TGA-3′ and 5′-TCC TGA TCG ACA AGA CCG GCT TCC-3′. As an internal control, the β-actin gene was also amplified using the same reaction protocol. The reaction was performed under standard conditions with 35 total cycles, each of which included denaturing at 94°C for 30 seconds, followed by 55°C for 30 seconds, 72°C for 45 seconds, and final extension at 72°C for 10 minutes. Amplified fragments were separated by electrophoresis on 2% agarose gel and visualized with ethidium bromide.

IL-7 Production Assay

IL-7 production capacity was tested in producing cells and transduced MSCs. After transduction with the vector containing IL-7 cDNA, cells were seeded in 6 well plates containing 1 mL of complete medium (DMEM + NCS 10%). After 24 and 48 hours of culture, the supernatant (corresponding to 100% confluence) was centrifuged and stored at −20°C until IL-7 production was determined using 2 different enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN; Beckman Coulter), according to the manufacturer's instructions. Results are expressed as pg/mL.

Naive T cells: Isolation

Blood samples were collected from healthy donors; peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifuge. The $CD45RA^+CD45RO^-$ population was isolated by a round of negative immunoselection with magnetic beads (Dynal, Lake Success, NY), in accordance with the manufacturer's instructions. Briefly, goat anti-mouse magnetic beads were preincubated with mouse anti-human CD45RO antibody (PharMingen, San Diego, CA) for 30 minutes at room temperature. Antibody-coated beads were then incubated with PBMCs for 30 minutes at 4°C [35].

Naive T Cells: In Vitro Culture

Naive ($CD3^+CD45RA^+$) enriched T cells were cultured for 7 days in 0.2 mL of RPMI-1640 medium (GIBCO BRL, Grand Island, NY) supplemented with 10% heat-inactivated human AB serum (Sigma, St Louis, MO), 1% penicillin/streptomycin (GIBCO-BRL), and 2 mmol of L-glutamine (GIBCO-BRL). Cells were cultured (a) alone and with (b) 4 μg/mL of phytohemagglutenin (PHA), (c) 0.01 ng/mL of human IL-7, (d) 100 ng/mL of human IL-7, (e) untransduced MSCs, or (f) MSCs engineered with the IL-7 gene. We assessed the activity of MSC-produced IL-7 by evaluating maintenance of the naive T-cell phenotype, apoptosis rate, and cell cycle. Cell phenotype was determined using a panel of monoclonal antibodies directed against the following antigens: CD3, CD4, CD8, CD14, CD25, CD71, CD45RA, and CD45RO (Beckman Coulter) and CCR7 (Becton Dickinson Bioscience, San Jose, CA) using a direct fluorescence labeling method. Cells were analyzed using an Epics XL cytometer (Beckman Coulter) on days 0, +1, and +7 of culture. Apoptosis was detected by determining phosphatidylserine exposure by annexin V binding using an Annexin V-FITC kit (Beckman Coulter) [36]. The cell cycle was assessed by flow cytometry analysis measuring DNA-bound propidium iodide fluorescence [37].

TCR V CDR3 Size Spectratyping

The CDR3 size distribution of 26 distinct TCR V families was determined by reverse-transcriptase PCR (RT-PCR) as described previously [38]. In brief, RNA was extracted and cDNA was synthesized using MMLV-RT (Amplimedical, Turin, Italy). PCR was performed with a forward primer specific for each of the 26 TCR V families along with a constant C reverse primer labelled with fluorescent FAM [39]. RT-PCR products were analyzed on an ABI PRISM 310 genetic analyzer using GeneScan software (Applied Biosystems, Foster City, CA). The normal TCR V CDR3 size was characterized by a Gaussian distribution containing 8-10 peaks for each V subfamily. The overall complexity of TCR V subfamilies was determined by spectratype scoring as described previously [40].

Elispot Assay

Naive T-cell production of interferon (IFN)-γ and IL-4 was determined in the presence of PHA (10 μg/mL final concentration) and nonengineered or engineered mesenchymal cells. PHA (10 μg/mL final concentration)-activated T lymphocytes were used as internal control. IFN-γ and IL-4 were determined at day +7 of culture using the ELISPOT kit (Amplimedical) according to the manufacturer's instructions.

The resulting spots were counted with an automatic Elispot reader.

Statistical Analysis

Data were analyzed using 1-factor analysis of variance to determine the significance of variations. Statistical significance was set at $< .05$. Results are expressed as mean ± standard deviation of 5 different experiments for each analysis.

RESULTS

Retroviral Vectors and Producing Cell Lines

GP+env+AM12/NeoR-pBabe/IL-7– and GP+env+AM12/NeoR-LXSN/IL-7– producing cell lines were obtained after ping-pong amplification, as described previously [41], in a titer of 1×10^6 cfu/mL of medium, as demonstrated by titration of the RAT-2 cell line. The vector-producing cell line was free of replication-competent viruses, as assessed by mobilization assay [42].

MSC Infection

Infections with both vectors were performed as described previously [31]. Briefly, a mean of 10,000 MSCs per mL of medium were transduced with GP+env+AM12/NeoR-pBabe/IL-7 and GP+env+AM12/NeoR-LXSN/IL-7 retroviral supernatants. After 10 days of G418 (0.6 mg/mL) selection, 95%–99% cells were infected with the NeoR gene [31]. No differences in infection rates were seen between the 2 vectors.

Crystal violet staining demonstrated no differences in cell morphology in untransduced, transduced/unselected, and transduced/selected MSCs with either vector. After transduction, the MSCs reformed the confluent layer with 100% viable cells at the confluence. Flow cytometry analysis revealed no significant differences in immunophenotypes of untransduced and transduced MSCs, demonstrating that the infection/selection procedures did not modify MSC surface expression of molecules. After 15 days of culture, MSCs engineered with both vectors were negative for CD45 and CD14 and positive for CD90, CD105, and STRO-1.

MSC Production of IL-7

ELISA assays on GP+env+AM12/NeoR-LXSN/IL-7 cells showed that they released 2000 pg/mL of IL-7 when the supernatant was collected after 24 hours of culture. pBabe retroviral vector-producing cells released around 60 pg/mL IL-7, as described previously [41]. In untransduced MSCs, IL-7 production was 0.6 pg/mL. At 30 days after infection, IL-7 production was 16 pg/mL in MSCs engineered with the pBabe packaging cell line and 1000 pg/mL in cells transduced using the LXSN packaging cell line. At 60 days after transduction, IL-7 production in MSC supernatant was unchanged, indicating stable transgene expression for both engineered MSC cultures.

Effects of IL-7–Transduced MSCs on Naive T-Cell Phenotype

After $CD45RO^+$-negative immunoselection, PBMCs were enriched for $CD3^+CD45RA^+$ cells (83.9% ± 11.6%), with a low percentage of $CD3^+CD45RO^+$ cells (13.5% ± 1.9%). The percentage of $CD3^+CD4^+$-positive cells was 52.2% ± 1.3%, and that of $CD3^+CD8^+$-positive cells was 36.6% ± 4.1%. No monocytes were found in the final fraction (mean percentage of $CD14^+$ cells, 0.014%).

After 7 days of culture with transduced MSCs producing 16 pg/mL of IL-7, $CD3^+CD45RA^+$ expression did not differ significantly from the starting fraction (78% ± 8.2% vs 83.9% ± 11.6%; $P > .05$), and $CD3^+CD45RO^+$ percentage remained low (11.5% ± 1.9%). In the presence of transduced MSCs producing 1000 pg/mL of IL-7, $CD3^+CD45RA^+$ expression remained stable at 83.4% ± 13.4%, and the percentage of $CD3^+CD45RO^+$ increased slightly to 16.8% ± 10.2% ($P > .05$) (Figure 1). The percentage of $CD3^+CD45RA^+$ did not differ significantly between high and low IL-7–producing MSCs ($P > .05$). Treatment of the naive ($CD3^+CD45RA^+$) enriched T-cell population with either 0.01 ng/mL or 100 ng/mL of exogenous IL-7 did not modify $CD3^+CD45RA^+$ expression (78% ± 11% and 80% ± 7.7%; $P > .05$). $CD3^+CD45RO$ remained low (12.8% ± 4.5% and 13% ± 5.4%), as in the starting fraction.

When naive T cells were cultured with PHA as a control, the percentage of $CD3^+CD45RO^+$ increased to 40.4% ± 12.5% ($P < .05$). Interestingly, culturing

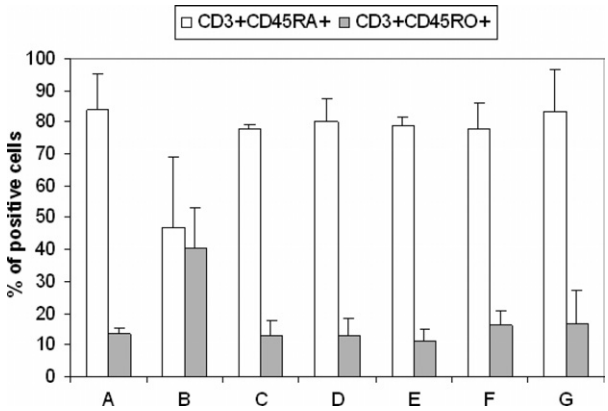

Figure 1. $CD3^+CD45RA^+$ and $CD3^+CD45RO^+$ expression on a $CD45RA^+$–enriched T-cell population on day 0 (A) and after 7 days of culture in the presence of PHA (B), 0.01 ng/mL of human IL-7 (C), 100 ng/mL of human IL-7 (D), untransduced MSCs (E), engineered MSCs producing 16 pg/mL of IL-7 (F), and engineered MSCs producing 1000 pg/mL of IL-7 (G).

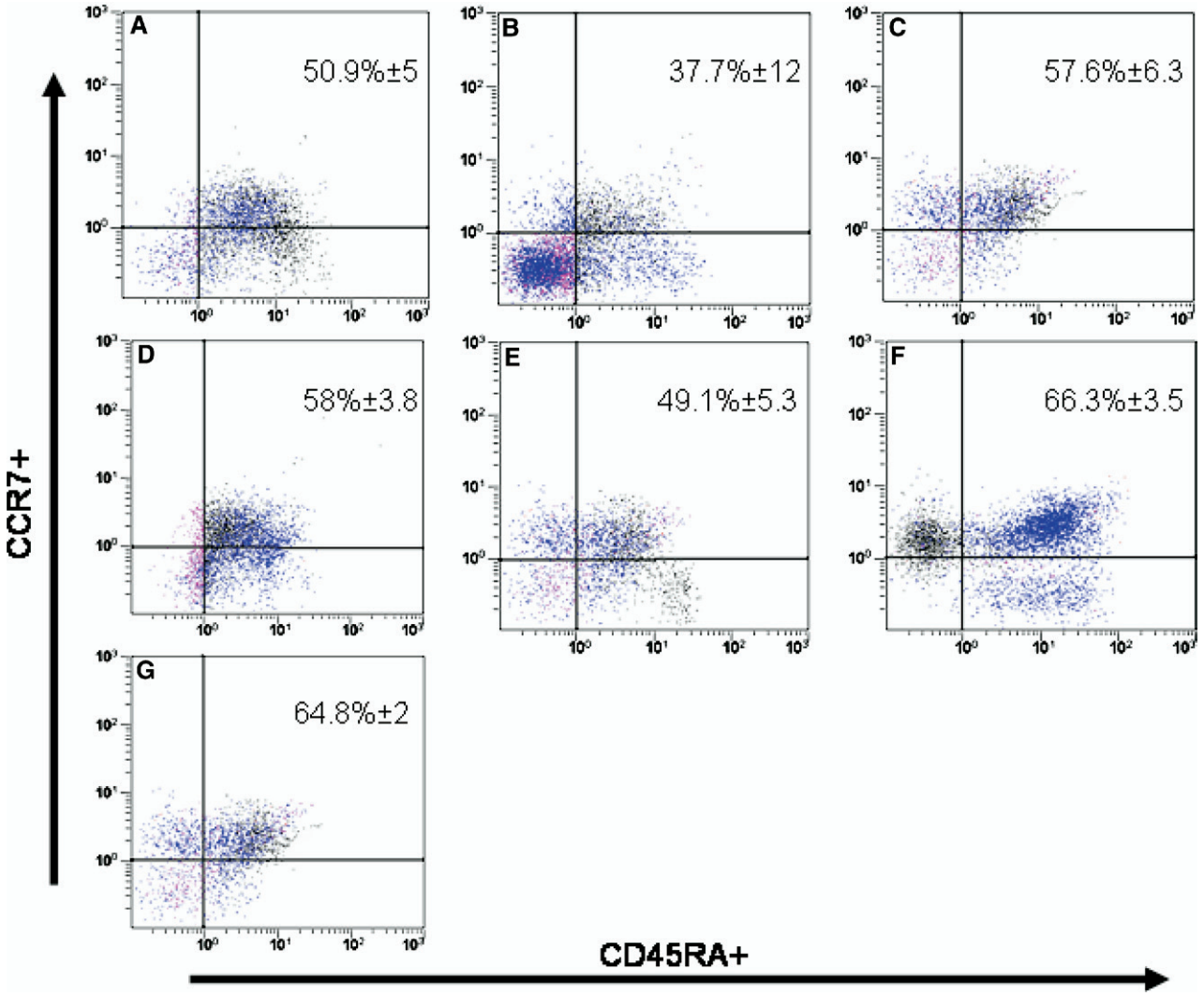

Figure 2. CCR7 expression patterns in $CD3^+CD45RA^+$. Expression of CD45RA and CCR7 after 7 days culture of naive T cells alone (A) and with PHA (B), 0.01 ng/mL of human IL-7 (C), 100 ng/mL of human IL-7 (D), untransduced mesenchymal cells (E), engineered MSCs producing 16 pg/mL of IL-7 (F), and engineered MSCs producing 1000 pg/mL of IL-7 (G).

the T-cell population with transcduced MSCs producing 1000 pg/mL of IL-7 produced a $CD45RA^+$/$CD45RO^+$ double-positive population of 8.8% ± 0.5%.

We analyzed the CCR7 expression patterns in the CD3/CD45RA and CD3/CD45RO populations after 7 days of culture under different conditions. The 50.9% ± 5% starting fraction of naive T cells characterized by co-expression of $CD3^+CD45RA^+CCR7^+$ rose to 66.3% ± 3.5% after 7 days of culture with IL-7 MSCs producing 16 pg/mL and to 64.8% ± 2% after culture with IL-7 MSCs producing 1000 pg/mL ($P < .05$ vs starting fraction for both). No significant difference in CD3/CD45RA/CCR7 expression was seen after culture with MSCs producing 16 pg/mL versus MSCs producing 1000 pg/mL ($P > .05$), demonstrating that different IL-7 dosages modified the naive T-cell phenotype in the same way (Figure 2). PHA reduced the percentage of $CD3^+CD45RA^+CCR7^+$ (37.7% ± 12%). IL-7 treatment of the enriched $CD3^+CD45RA^+$ population with either 0.01 ng/mL or 100 ng/mL of exogenous cytokine did not modify $CD3^+CD45RA^+CCR7^+$ expression (57.6% ± 6.3% and 58% ± 3.8%, respectively; $P > .05$ vs starting fraction).

The residual $CD3^+CD45RO^+$ memory T cells present in the $CD45RA^+$–enriched T-cell population were analyzed after culture only with transduced mesenchymal cells producing 1000 pg/mL of IL-7. We found that the $CD3^+CD45RO^+CCR7^+$ subset (central memory cells) increased slightly from a starting fraction of 1.5% ± 1.4% to 10.9% ± 6.9% ($P < .05$), whereas the $CD3^+CD45RO^+CCR7^-$ subset (effector memory cells) remained unchanged (starting fraction, 9.2% ± 6.6% vs 6.9% ± 4.9%; $P > .05$). Nonengineered MSCs (controls) increased the percentage of central memory cells slightly but did not affect the effector memory cells. PHA increased the percentage of central memory cells (from 1.5% ± 1.4% to 13.7%

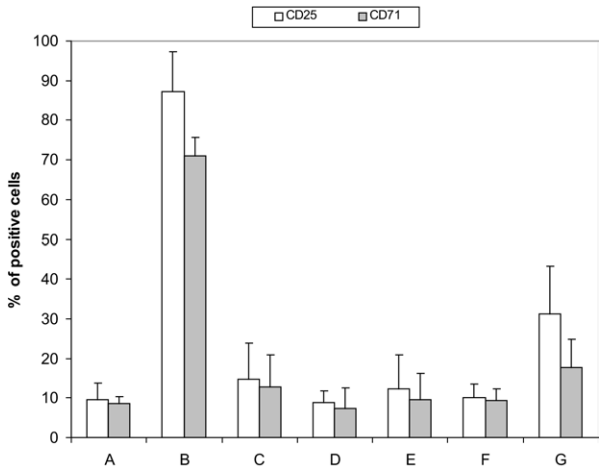

Figure 3. CD25/CD71 expression. Expression of CD25 and CD71 after 7 days culture of naive T cells alone (A) and with PHA (B), 0.01 ng/mL of human IL-7 (C), 100 ng/mL of human IL-7 (D), untransduced MSCs (E), engineered MSCs producing 16 pg/mL of IL-7 (F), and engineered MSCs producing 1000 pg/mL of IL-7 (G).

± 2.2%; $P < .05$), but not of effector memory cells (from 9.2% ± 6.6% to 7.8% ± 8.1%; $P > .05$).

Among the naive T-cell population, the percentages of $CD4^+$ and $CD8^+$ cells remained unvaried after 7 days of culture with engineered MSCs producing 16 pg/mL of IL-7, confirming previously reported results [31]. In co-culture with transduced MSCs producing 1000 pg/mL, the percentage of $CD8^+$ dropped to 25.8% ± 5.4% from a starting fraction of 36.1% ± 4.1% ($P > .05$), and the percentage of $CD4^+$ increased to 62.9% ± 10.3% from a starting fraction of 52.2% ± 1.3% ($P > .05$).

Effects of IL-7–Transduced MSCs on Activation and Proliferation Markers in Naive T Cells

Evaluation of CD25 and CD71 at 24 hours of culture revealed no significant change in surface expression molecules. Analysis of CD25 and CD71 expression of the naive enriched T-cell population after 7 days of culture under different culture conditions showed mean CD 25 and CD71 expression in naive T cells alone of 9.7% ± 4% and 8.7% ± 1.5%, respectively. As a control, the PHA treatment of naive T cells up-regulated the CD25 and CD71 expression to 87.3% ± 10% and 71% ± 4.6% ($P < .05$), respectively. In naive T cells cultured with 0.01 ng/mL of IL-7, CD25 expression was 14.7% ± 9.2% and CD71 expression was 12.7% ± 8.3%; in 100 ng/mL of IL-7, these values were 8.8% ± 3.1% and 7.35% ± 5.1%, respectively ($P > .05$ for both).

In $CD45RA^+$–enriched T-cells co-cultured with untransduced MSCs, CD25 expression was 12.4% ± 8.4% ($P > .05$) and CD71 expression was 9.7% ± 6.4% ($P > .05$). The naive T-cell population co-cultured with engineered MSCs producing 16 pg/mL of IL-7 did not modify CD25 and CD71 expression (10% ± 3.5% and 9.3% ± 3%,respectively) compared with naive T cells alone ($P > .05$). In naive enriched T cells co-cultured with engineered MSCs producing 1000 pg/mL of IL-7, CD25 and CD71 expression was up-regulated to 31.2% ± 12% and 17.8% ± 7%, respectively ($P < .05$ vs naive T cells alone, vs naive T cells cultured with r-IL-7, vs untransduced MSCs, and vs MSCs producing 16 pg/mL of IL-7) (Figure 3). The engineered MSCs maintained significantly higher naive T-cell counts after 7 days of culture of naive T cells alone, naive T cells with 0.01 or 100 ng/mL of human recombinant IL-7, or untransduced stromal cells [31]. Despite a trend toward significance with high-dose IL-7 MSCs, a comparison of high-dose and low-dose IL-7–engineered MSCs revealed no significant difference in naive T-cell counts.

Effects of IL-7–Transduced Mesenchymal Cells on Naive T-Cell Apoptosis

The apoptosis rate of naive T cells cultured with engineered MSCs producing 1000 pg/mL of IL-7 increased to 18.6 ± 5, compared with 14.1 ± 6.39 ($P > .05$) in freshly isolated cells and 14.9 ± 2.6 ($P > .05$) in engineered MSCs producing 16 pg/mL of IL-7. As expected, the apoptosis rate increased to 27.33 ± 7.5 in the PHA-treated enriched T cells (Figure 4). Interestingly, exogenous IL-7 treatment of $CD3^+CD45RA^+$–enriched T cells at either 0.01 or 100 ng/mL did not significantly increase the apoptosis rate (13.6 ± 7.5 vs 11.8 ± 3.3; $P > .05$). No significant differences in necrosis and secondary apoptosis rates were seen (data not shown).

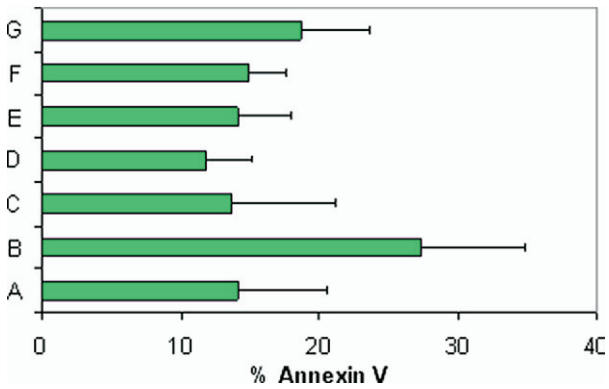

Figure 4. Apoptosis and cell cycle analyses: Apoptosis rates expressed as percentage of annexin V on a $CD45RA^+$–enriched T-cell population after 7 days of culture of T cells alone (A) and in the presence of PHA (B), 0.01 ng/mL of human IL-7 (C), 100 ng/mL of human IL-7 (D), untransduced MSCs (E), engineered MSCs producing 16 pg/mL of IL-7 (F), and engineered MSCs producing 1000 pg/mL of IL-7 (G).

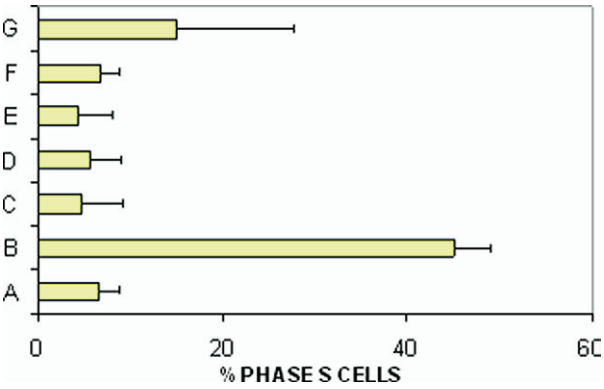

Figure 5. Apoptosis and cell cycle analyses: Cell cycle phase S progression of a CD45RA$^+$–enriched T-cell population after 7 days of culture of T cells alone (A) and in the presence of PHA (B), 0.01 ng/mL of human IL-7 (C), 100 ng/mL of human IL-7 (D), untransduced MSCs (E), engineered MSCs producing 16 pg/mL of IL-7 (F), and engineered MSCs producing 1000 pg/mL of IL-7 (G).

Effects of IL-7–Transduced MSCs on Naive T-Cell Cycle Progression

Cell cycle analysis showed that the percentage of phase S cells was 6.5% ± 2.4% when the CD45RA$^+$–enriched T-cell population was cultured alone for 7 days. PHA-induced cell cycle progression, with approximately 45% ± 4.2% of the T cells entering the S phase of the cell cycle. IL-7 treatment with either low or high exogenous cytokine was similar to untreated culture, with only 4.7% ± 1% and 4.4% ± 3% cells entering the S phase of the cell cycle. The percentage did not change significantly when naive T cells were co-cultured with nonengineered MSCs or with engineered MSCs producing 16 pg/mL of IL-7 (4.4% ± 2% vs 6.9% ± 1%; $P > .05$). With engineered MSCs producing 1000 pg/mL of IL-7, the percentage of cells entering the S phase rose to 15% ± 3% ($P > .05$ vs mesenchymal cells producing 16 pg/mL of IL-7) (Figure 5).

Effects of IL-7–Transduced MSCs on Naive T- Cell Expression of Type 1/Type 2 Cytokines

The effects of engineered and nonengineered MSCs on naive T-cell differentiation to Th1 or Th2 was determined by assessing secretion of IFN-γ and IL-4, respectively, after 7 days of culture. No cytokine accumulation was detected. PHA used as a positive control exhibited cytokine accumulation in 50 spots.

Effects of IL-7–Transduced MSCs on Naive TCR V CDR3 Size Spectratyping

The V spectratyping complexity score of T cells after 7 days of culture with IL-7–engineered MSCs did not differ significantly from the starting fraction (174 and 155 vs 167; $P > .05$), indicating a broad T-cell repertoire (Figure 6).

DISCUSSION

The present results demonstrate that MSCs engineered to produce IL-7 exert a dose-dependent effect on CD3$^+$CD45RA$^+$ naive T-cell activation/proliferation and a dose-independent effect on survival. When naive T cells were co-cultured with engineered MSCs producing 16 pg/mL of IL-7, the CD45RA$^+$CD45RO$^-$ phenotype was maintained, confirming previous findings that IL-7 does not lead to CD45RO up-regulation [43-46]. Interestingly, when the enriched T-cell population was co-cultured

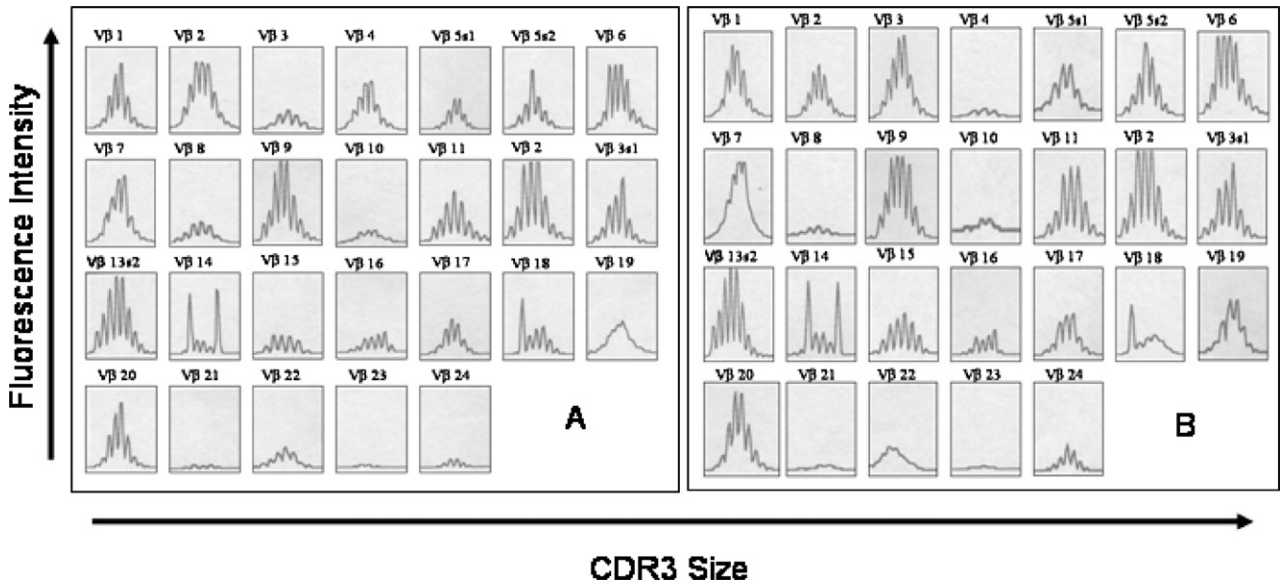

Figure 6. V spectratyping complexity score. The CDR3 size distribution of 26 distinct TCR V families was determined on a CD45RA$^+$–enriched T-cell population on day 0 (A) and after 7 days of culture with engineered MSCs producing 1000 pg/mL of IL-7.

with MSCs producing 1000 pg/mL of IL-7, the naive T-cell phenotype was maintained, as demonstrated by $CD3^+CD45RA^+CCR7^+$ expression, lack of Th1 or Th2 differentiation, and a broad T-cell repertoire. At the same time, increases were found in T-cell proliferation/activation markers, apoptosis rate, and cell cycle progression.

Several in vitro studies have reported conflicting results after administration of different dosages of IL-7 to naive adult or cord blood T cells or to thymocytes. After 7 days of treatment with 1000 U/mL of IL-7, naive T cells proliferated without acquiring the primed CD45RO phenotype [35]. In vitro doses of 1-100 ng/mL of human recombinant IL-7 did not induce naive T-cell proliferation, but did prevent cell death and atrophy [47]. Adding 5 ng/mL of IL-7 to naive adult and cord blood T cells prevented apoptosis and induced cell cycling in up to 20% of naive T cells [43]. After treatment with 10 ng/mL of exogenous IL-7, naive adult peripheral blood T cells did not divide [48]. A recent report claimed that exogenous administration of IL-7 had no significant effect on immune reconstitution in major histocompatibility complex–compatible and partially incompatible settings [49].

A key difference between the present study and the studies cited above is the IL-7 delivery system. In our in vitro model, MSC-mediated delivery of IL-7 was more efficacious than exogenous administration, probably because T-cell precursor development depends not only on cytokines, but also on interactions between thymocyte precursors and stromal cells, which involve cell surface molecules, extracellular matrix (ECM) components, and soluble growth factors [50]. IL-7–engineered MSCs present IL-7 in a bioactive state, which, through MSC expression of ECM components, may modulate adhesive interactions with naive T cells [51].

Evidence in support of these findings comes from our observation of a dose-dependent effect of the IL-7–engineered MSCs on proliferation of $CD45RA^+$–enriched T-cells. MSCs producing 16 pg/mL of IL-7 were associated with a low proliferation profile, whereas MSCs engineered to produce 1000 pg/mL of IL-7 up-regulated activation/proliferation markers (CD25/CD71) and increased the percentage of cells in phase S. Interestingly, Managlia et al [52] reported that IL-7 up-regulated activation markers (CD25/CD71) but did not induce cell cycle proliferation of $CD4^+CD45RA^+CD45RO^-$ T cells. Again, different doses and delivery systems may account for the differences.

The IL-7 dose–dependent effect is illustrated by apoptosis rates of $CD45RA^+$–enriched T-cells in the presence of 2 different IL-7–engineered MSC types. Low IL-7 concentrations are associated with a low apoptosis rate, whereas high IL-7 levels tend to increase apoptosis, in contrast to other observations indicating that IL-7 maintains naive T-cell survival. IL-7 has been reported to prevent cell death and atrophy of naive T cells independent of dosage [47]. Through transduction, MSCs may improve the ECM-mediated presentation of IL-7 to naive T cells, likely facilitating use of cytokine by T-cell precursors and enhancing activation, proliferation, and apoptosis.

Strangely, the dose-dependent effect was not confirmed by differentiation to either Th1 or Th2 phenotypes, because even the high IL-7 concentration was not sufficient to induce differentiation. This observation confirms that IL-4 and IFN-γ naive T-cell expression remains minimal after phorbol 12-myristate 13-acetate (PMA) or ionomycin stimulation even after IL-7 stimulation [53]. On the other hand, Managlia et al [52] found that the IL-7–treated $CD4^+CD45RA^+CD45RO^-$ T cells produce IFN-γ but not IL-4. Spectratyping analysis of the T-cell repertoire complexity indicated that IL-7–engineered MSCs did not exert a dose-dependent effect, because a broad T-cell repertoire was always maintained.

One could hypothesize that our in vitro system reproduces 2 in vivo models illustrating IL-7–related modulation of peripheral T-cell homeostasis [4]. "Homeostatic cycling" describes naive or memory cell turnover in T-cell–replete hosts, which does not modify the functional profile of the cycling population. In our model, engineered MSCs producing low IL-7 concentrations induce homeostatic cycling without altering the naive phenotype. "Homeostatic peripheral expansion" refers to T-cell expansion in T-cell–depleted hosts, which is associated with naive T cells developing into memory T cells. In our system, although engineered MSCs producing high IL-7 concentrations do not switch the $CD45RA^+$ phenotype to a $CD45RO^+$ phenotype, they do modify other proliferation/activation parameters. Whether these modifications signify homeostatic peripheral expansion is a matter of debate.

In our model, when naive enriched T cells were cultured with IL-7–engineered MSCs, the $CD3^+CD45RA^+CCR7^+$ population, which identifies the naive phenotype, remained unchanged. When we analyzed the effects of engineered MSCs producing 1000 pg/mL of IL-7 on the $CD45RO^+$ residual subpopulation in the $CD45RA^+$–enriched T-cell population, we observed that the central memory cell subset ($CD3^+CD45RO^+CCR7^+$) expanded more than the effector memory cell subset ($CD3^+CD45RO^+CCR7$-). Our results do not concur with previous findings that exogenous IL-7 administration expands the effector memory cell subset more than the central memory cell subset [54]; however, that previous study analyzed the effects of IL-7 on $CD4^+$ cells, whereas the present investigation analyzed the effects on $CD4^+$ cells and the CD8 subset.

In conditions of T-cell depletion, naive T cells undergo spontaneous "homeostatic" proliferation when exposed to certain cytokines [55-57]. The efficacy of these cytokines in driving cell survival and homeostatic proliferation appears to differ in $CD4^+$ and $CD8^+$ cells. In fact, although exogenous IL-4, IL-7, and IL-15 enhance homeostatic proliferation of naive $CD8^+$ cells in vitro, $CD4^+$ cells are less sensitive. Exposure to exogenous IL-7 expands the $CD8^+$ subpopulation more than the $CD4^+$ subpopulation [35], and the present study shows that MSCs engineered to express high IL-7 concentrations expand the $CD8^+$ and the $CD4^+$ subpopulations in the same way.

Insertional mutagenesis is a major risk factor associated with virus-based vector systems [58], and efforts are being made to alleviate this problem. For example, alterations within the lentiviral vector long-terminal repeats are being created to disable enhancer activity and reduce the potential for interference with host gene expression after transgene integration [59]. An alternative approach is to develop expression systems that, because they are designed as sustained nonviral, nonintegrated vectors, are free of major safety problems [60]. Should continuous IL-7 secretion from MSCs increase the risk of lymphoproliferative disease, co-transduction with a suicide gene, such as the thymidine kinase gene, will eliminate unwanted cells by treatment with ganciclovir [61].

In conclusion, MSCs engineered to produce IL-7 at low and high concentrations maintain the naive T-cell phenotype ($CD3^+CD45RA^+CCR7^+$) with no up-regulation of $CD45RO^+$ and no changes in V beta spectratyping. High IL-7–producing MSCs enhance apoptosis and increase the number of T cells in the phase S cell cycle and activation/proliferation markers in the naive enriched T-cell population. Therefore, IL-7 produced by MSCs has a dose-independent effect on naive T-cell survival and a dose-dependent effect on proliferation. Administering high-dose IL-7–engineered MSCs may hasten de novo T-cell reconstitution after allogeneic bone marrow transplantation.

ACKNOWLEDGMENT

The authors thank Dr. Geraldine Anne Boyd for her help with this study. This work was supported by the Associazione Umbra Leucemie e Linfomi, Grifo Latte Perugia, and Rotary Club Perugia Est Perugia. Paolo Sportoletti, Beatrice Del Papa, Antonio Tabilio, and Mauro Di Ianni contributed equally to this work.

REFERENCES

1. Michie CA, McLean A, Alcock C, et al. Lifespan of human lymphocyte subsets by CD45 isoforms. *Nature*. 1992;360:264-265.
2. Fry TJ, Mackall CL. Interleukin-7: from bench to clinic. *Blood*. 2002;99:3892-3904.
3. Peschon JJ, Morrissey PJ, Grabstein KH, et al. Early lymphocyte expansion is severely impaired in interleukin-7 receptor–deficient mice. *J Exp Med*. 1994;180:1955-1960.
4. Fry TJ, Mackall CL. The many faces of IL-7: from lymphopoiesis to peripheral T-cell maintenance. *J Immunol*. 2005;174:6571-6576.
5. Von Freeden-Jeffry U, Vieira P, Lucian LA, et al. Lymphopenia in interleukin (IL-7) gene–deleted mice identifies IL-7 as a nonredundant cytokine. *J Exp Med*. 1995;181:1519-1526.
6. Puel A, Ziegler SF, Buckley RH, et al. Defective IL7R expression in T(−)B(+)NK(+) severe combined immunodeficiency. *Nat Genet*. 1998;20:394-397.
7. Tan JT, Dudl E, Leroy E, et al. IL-7 is critical for homeostatic proliferation and survival of naive T cells. *Proc Natl Acad Sci USA*. 2001;98:8732-8737.
8. Marrack P, Kappler J. Control of T-cell viability. *Annu Rev Immunol*. 2004;33:765-787.
9. Kim K, Lee CK, Sayers TJ, et al. The trophic action of IL-7 on pro-T cells: inhibition of apoptosis of pro-T1, -T2, and -T3 cells correlates with Bcl-2 and Bax levels and is independent of Fas and p53 pathways. *J Immunol*. 1998;160:5735-5741.
10. Park JH, Yu Q, Erman B, et al. Suppression of IL7R-alpha transcription by IL-7 and other pro-survival cytokines: a novel mechanism for maximizing IL-7–dependent T-cell survival. *Immunity*. 2004;21:289-302.
11. Kaech SM, Ahmed R. Memory $CD8^+$ T-cell differentiation: initial antigen encounter triggers a developmental program in naive cells. *Nat Immunol*. 2001;2:415-422.
12. Kieper WC, Jameson SC. Homeostatic expansion and phenotypic conversion of naive T cells in response to self peptide/MHC ligands. *Proc Natl Acad Sci USA*. 1999;96:13306-13311.
13. Schluns KS, Kieper WC, Jameson SC, et al. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. *Nat Immunol*. 2000;1:426-432.
14. Fry TJ, Connick E, Falloon J, et al. A potential role for interleukin-7 in T-cell homeostasis. *Blood*. 2001;97:2983-2990.
15. Bhandoola A, Tai X, Eckhaus M, et al. Peripheral expression of self MHC-II influences the reactivity and self-tolerance of mature CD4(+) T cells: evidence from a lymphopenic T-cell model. *Immunity*. 2002;17:425-436.
16. Alpdogan O, Schmaltz C, Muriglan SJ, et al. Administration of interleukin-7 after allogeneic bone marrow transplantation improves immune reconstitution without aggraving graft-versus-host desease. *Blood*. 2001;98:2256-2265.
17. Sinha ML, Fry TJ, Fowler DH, et al. Interleukin-7 worsens graft-versus-host disease. *Blood*. 2002;100:2642–2549.
18. Smithgall MD, Wong JG, Critchett KE, et al. IL-7 up-regulates HIV-1 replication in naturally infected peripheral blood mononuclear cells. *J Immunol*. 1996;156:2324-2330.
19. Broers AE, Posthumus-Van Slujis SJ, Spits H, et al. Interleukin-7 improves T-cell recovery after experimental T-cell–depleted bone marrow transplantation in T-cell–deficient mice by strong expansion of recent thymic emigrants. *Blood*. 2003;102:1534-1540.
20. Levy RB, Jones M, Hamilton BL, et al. IL-7 drives donor T-cell proliferation and can costimulate cytokine secretion after MHC-matched allogeneic bone marrow transplantation. *J Immunol*. 1995;154:106-115.
21. Abdul-Hai A, Or R, Slavin S, et al. Stimulation of immune

reconstitution by interleukin-7 after syngeneic bone marrow transplantation in mice. *Exp Hematol*. 1996;24:1416-1422.
22. Bolotin E, Smogorzewska M, Smith S, et al. Enhancement of thymopoiesis after bone marrow transplant by in vivo interleukin-7. *Blood*. 1996;88:1887-1894.
23. Alpdogan O, Muriglan SJ, Eng JM, et al. IL-7 enhances peripheral T-cell reconstitution after allogeneic hematopoietic stem cell transplantation. *J Clin Invest*. 2003;112:1095-1107.
24. Mackall CL, Fry TJ, Bare C, et al. IL-7 increases both thymic-dependent and thymic-independent T-cell regeneration after bone marrow transplantation. *Blood*. 2001;97:1491-1497.
25. Deans RJ, Moseley AB. Mesenchymal stem cells: biology and potential clinical uses. *Exp Hematol*. 2000;28:875-884.
26. Bartholomew A, Patil S, Mackay A, et al. Baboon mesenchymal stem cells can be genetically modified to secrete human erytropoietin in vivo. *Hum Gene Ther*. 2001;12:1527-1541.
27. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. *Science*. 1999; 284:143-147.
28. Verfaille CM. Adhesion receptors as regulators of the hematopoieic process. *Blood*. 1998;92:2609-2612.
29. Majumdar MK, Thiede MA, Mosca JD, et al. Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSCs) and stromal cells. *J Cell Physiol*. 1998;176:57-66.
30. Barda-Saad M, Rozenszajn LA, Globerson A, et al. Selective adhesion of immature thymocytes to bone marrow stromal cells: relevance to T-cell lymphopoiesis. *Exp Hematol*. 1996;24: 386-391.
31. Di Ianni M, Del Papa B, De Ioanni M, et al. Interleukin-7–engineered stromal cells: a new approach for hastening naive T-cell recruitment. *Hum Gene Ther*. 2005;16:752-764.
32. Wu BW, Lu J, Gallaher TK, et al. Identification of regions in the Moloney murine leukemiavirus SU protein that tolerate the insertion of an integrin-binding peptide. *Virology*. 2000;269:7-17.
33. Di Ianni M, Casciari C, Ciurnelli R, et al. Beta-galactosidase–transduced T lymphocytes: a comparison between stimulation by either PHA and IL-2 or a mixed lymphocyte reaction. *Haematologica*. 1996;81:410-417.
34. Cilloni D, Carlo-Stella C, Falzetti F, et al. Limited engraftment capacity of bone marrow–derived mesenchymal cells following T-cell–depleted hematopoietic stem cell transplantation. *Blood*. 2000;96:3637-3643.
35. Steffens CM, Managlia EZ, Landay A, et al. Interleukin-7–treated naive T cells can be productively infected by T-cell–adapted and primary isolated of human immunodeficiency virus 1. *Blood*. 2002;99:3310-3318.
36. Vermes I, Haanen C, Reutelingsperger C. Flow cytometry of apoptotic cell death. *J Immunol Meth*. 2000;243:167-190.
37. Gerashchenko BI, Azzam EI, Howell RW. Characterization of cell cycle progression and growth of WB-F344 normal rat liver epithelial cells following gamma-ray exposure. *Cytometry A*. 2004;61:134-141.
38. Eiraku N, Hingorani R, Ijichi S, et al. Clonal expansion within $CD4^+$ and $CD8^+$ T-cell subsets in human T lymphotropic virus type I–infected individuals. *J Immunol*. 1998;161:6674-6680.
39. Gorsky J, Piatek T, Yassai M, et al. Improvements in repertoire analysis by CDR3 size spectratyping: bifamily PCR. *Ann NY Acad Sci*. 1995;756:99-102.
40. Wu CJ, Chillemi A, Alyea EP, et al. Reconstitution of T-cell receptor repertoire diversity following T-cell–depleted allogeneic bone marrow transplantation is related to hematopoietic chimerism. *Blood*. 2000;95:352-359.
41. Di Ianni M, Del Papa B, De Ioanni M, et al. Mesenchymal cells: a vehicle for gene therapy. *Blood Cells Mol Dis*. 2004;33:267-270.
42. Varmus H. Retroviruses. *Science*. 1988;240:1427-1435.
43. Soares MV, Borthwick NJ, Maini MK, et al. IL-7–dependent extrathymic expansion of $CD45RA^+$ T cells enables preservation of a naive repertoire. *J Immunol*. 1998;161:5909-5917.
44. Cavalieri S, Cazzaniga S, Geuna M, et al. Human T lymphocytes transduced by lentiviral vectors in the absence of TCR activation maintain an intact immune competence. *Blood*. 2003; 102:497-505.
45. Scripture-Adams DD, Brooks DG, Korin YD, et al. Interleukin-7 induces expression of latent human immunodeficiency virus type 1 with minimal effects on T-cell phenotype. *J Virol*. 2002;76:13077-13082.
46. Webb LM, Foxwell BM, Feldmann M. Putative role for interleukin-7 in the maintenance of the recirculating naive $CD4^+$ T-cell pool. *Immunology*. 1999;98:400-405.
47. Rathmell JC, Farkash EA, Gao W, et al. IL-7 enhances the survival and maintains the size of naive T cells. *J Immunol*. 2001;167:6869-6876.
48. Dardalhon V, Jaleco S, Kinet S, et al. IL-7 differentially regulates cell cycle progression and HIV-1–based vector infection in neonatal and adult $CD4^+$ T cells. *Proc Natl Acad Sci USA*. 2001;98:9277-9282.
49. Andre-Schmutz I, Bonhomme D, Yates F, et al. IL-7 effect on immunological reconstitution after HSCT depends on MHC incompatibility. *Br J Haematol*. 2004;126:844-851.
50. Banwell CM, Parkington KM, Jenkinson EJ, et al. Studies on the role of IL-7 presentation by mesenchymal fibroblasts during early thymocyte development. *Eur J Immunol*. 2000;20: 2125-2129.
51. Clarke D, Katoh O, Gibbs RV, et al. Interaction of interleukin 7 (IL-7) with glycosaminoglycans and its biological relevance. *Cytokine*. 1995;7:325-330.
52. Managlia EZ, Landay A, Al-Harthi L. Interleukin-7 signalling is sufficient to phenotypically and functionally prime human CD4 naive T cells. *Immunology*. 2005;114:322-335.
53. Verhoeyen E, Dardalhon V, Ducrey-Rundquist O, et al. IL-7 surface-engineered lentiviral vectors promote survival and efficient gene transfer in resting primary T lymphocytes. *Blood*. 2003;101:2167-2174.
54. Geginat J, Sallusto F, Lanzavecchia A. Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD(+) T cells. *J Exp Med*. 2001;194: 1711-1719.
55. Surh CD, Sprent J. Homeostatic T-cell proliferation: how far can T cells be activated to self-ligands? *J Exp Med*. 2000;192: F9-F14.
56. Goldrath AW, Luckey CJ, Park R, et al. The molecular program induced in T cells undergoing homeostatic proliferation. *Proc Natl Acad Sci USA*. 2004;101:16885-16890.
57. Goldrath AW, Bevan MJ. Selecting and maintaining a diverse T-cell repertoire. *Nature*. 1999;402:255-262.
58. Hacein-Bey-Abina S, Von Kalle C, Schmidt M, et al. LMO-2 associated clonal T-cell proliferation in two patients after gene therapy for SCID-X1. *Science*. 2003;302:415-419.

59. Gaspar HB, Parsley KL, Howe S, et al. Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector. *Lancet*. 2004;364:2181-2187.
60. Jackson DA, Juranek S, Lipps HJ. Designing nonviral vectors for efficient gene transfer and long-term gene expression. *Mol Ther*. In press.
61. Di Ianni M, Terenzi A, Falzetti F, et al. Homing and survival of thymidine kinase-transduced human T cells in NOD/SCID mice. *Cancer Gene Ther*. 2002;9:756-761.