Human Mature T Cells That Are Anergic In Vivo Prevail in SCID Mice Reconstituted with Human Peripheral Blood

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Summary

In these studies we have characterized the human cells that repopulate severe combined immunodeficient (SCID) mice after injection of adult peripheral blood or cord blood (hu-PBL-SCID mice). In all organs of the chimeras, and at any time point tested, single-positive (CD4$^+$ or CD8$^+$) T cells that expressed the α/β T cell receptor (TCR) prevailed. All T cells were CD45RO$^+$ and the majority were also HLA-DR$^+$. Thus, the human T cells in the chimeras exhibited the phenotype of mature, memory cells that showed signs of recent activation. Cell cycle studies revealed a mitotically active human T cell population in the murine host. However, when freshly isolated from the chimeras, the human T cells were refractory to stimulation by anti-CD3 antibody but proliferated in response to exogenous interleukin 2. Chimera-derived human T cell lines retained this state of unresponsiveness to TCR-triggered proliferation for 4–6 wk in vitro. Subsequently, the T cell lines developed responses to anti-CD3 stimulation and 9 of 11 of the lines also proliferated in response to splenic stimulator cells of SCID mice. These data demonstrate that the human T cells are in a state of reversible anergy in the murine host and that xenoreactivity might play a critical role in hu-PBL-SCID mice. Mechanisms that may determine repopulation of SCID mice with human peripheral blood mononuclear cells are discussed.

The CB.17 *scid/scid* (SCID) mouse strain exhibits defective recombination of antigen receptor genes (1) leading to an early arrest in T and B lymphocyte development and a resulting SCID. Therefore, SCID mice are unable to reject allogeneic or xenogeneic transplants (2). This notion prompted the idea that SCID mice could be engrafted with human leukocytes and hematopoietic cells to constitute a chimera with a functional human immune system (3, 4). Such SCID mouse human chimeras would be of particular interest for studies on human lymphocyte development and functions in vivo in a readily accessible animal model.

Construction of a functional human immune system in the SCID mouse has been pursued by introducing human lymphopoiesis into the mouse by grafting fetal human thymus, liver, and lymph nodes (4), or adult bone marrow (5); and transferring immunocompetent lymphocytes from peripheral blood into the mouse (hu-PBL-SCID mice) (3). The first approach resulted in long-term human lymphopoiesis in the fetal human graft (6). Mature, human T cells appeared in low numbers in the circulation of the mice and proved to be phenotypically normal and functional when tested in vitro (7). However, it is unclear whether these T cells also could

respond to antigen in the chimeras in the absence of human APC. Macrophages have not been detected in the chimeras (7) and B cells are poor APC for initiation of T cell responses (8–10). Therefore, SCID mice transplanted with fetal human lymphopoietic organs might not provide experimental animals with a fully functional immune system.

Theoretically, injection of SCID mice with human PBL transfers all the mature cell lineages required to constitute a functional immune system. High levels of human Ig were detected in such mice, indicating that mature human B lymphocytes repopulated and functioned in the recipients (3, 11). However, the human B cells were present in the chimeras in low numbers and these B cells exhibited a rather limited clonal heterogeneity (11). Furthermore, human T cells in hu-PBL-SCID mice had been detected infrequently and in low numbers and were, therefore, hard to analyze (12). With the human lymphocytes being at or beyond the detection limit, the nature of hu-PBL-SCID chimerism has not yet been defined.

Here we phenotypically and functionally characterize the human cells that populate hu-PBL-SCID chimeras. We report that 1 to 2 mo after intraperitoneal injection of SCID

mice with human PBL or cord blood, human lymphocytes become detectable by FACS® analysis and eventually constitute up to 50% of all mononuclear cells in the lungs, liver, and spleen of the recipient mice. Except for low numbers of B cells, the human leukocytes consist of mature T lymphocytes which exhibit the phenotype of activated memory cells. The human lymphocytes proliferate in their secondary host. However, we find that the human T cells in the chimeras are refractory to stimulation through the TCR but proliferate in response to IL-2. Human T cell lines established from hu-PBL-SCID mice retain this state of unresponsiveness during the first weeks of culture; subsequently, the lines become functional and proliferate to SCID-splenic stimulator cells.

Materials and Methods

Subjects. PBMC donors were 11 healthy subjects (eight men and three women) between the ages of 22 and 45 yr. Three of these donors had not been infected with EBV as judged by a lack of serum antibodies for EBV and by gene amplification to EBV genome (13, 14) using their PBMC DNA (see below). Cord blood from six newborns was kindly provided by Y. J. Bryson, UCLA School of Medicine. Four normal subjects provided bone marrow (one was EBV seronegative and genome negative). Thymus specimens were obtained in the course of cardiac surgery from two children under 5 yr of age. The thymus cell suspension was kindly provided by C. H. Uittenbogaart, UCLA School of Medicine. A human, immature T leukemia cell line, CCRF-CEM (CEM), grown continuously in serum-free medium, was provided by C. H. Uittenbogaart.

Mice and Cell Transfer. CB.17 SCID mice were bred at UCLA under sterile conditions and maintained in a pathogen-free environment without prophylactic antibiotic treatment. This colony, begun from founders delivered by cesarian section, has been screened for the presence of mouse pathogens. SCID mice were between 6 and 12 wk old at the time of cell transfer. PBMC and cord blood cells were isolated from blood by density gradient centrifugation (Lymphoprep; Nycomed Pharma AS, Oslo, Norway). Bone marrow was used either unmanipulated or as mononuclear cell suspension after Lymphoprep separation. The human cells were injected intraperitoneally in 1 ml of PBS into SCID mice via a 23-gauge needle. The number of cells injected in each experiment is indicated in the results. No mice developed frank human lymphoid tumors when observed up to 200 d after human cell transfer.

Human Ig Measurements. After transplantation with human cells, mice were bled every 2 wk from the tail vein and human serum Ig levels were measured by isotype-specific ELISAs as described in detail elsewhere (15). The detection limit of the assay for human IgG was 2.5 ng/ml. Hu-PBL-SCID mice sera were usually diluted between 1:25,000 and 1:250,000. Sera from naive SCID mice and BALB/c mice gave no measurable human Ig signal in the ELISAs, even at a dilution of 1:20.

Recovery of Human Cells from Grafted SCID Mice. Heparinized blood was collected from the tail vein of mice. Subsequently, the mice were killed by cervical dislocation and the spleen, liver, thymus, lymph nodes, and lungs were collected. Single cell suspensions of these organs were obtained by gently teasing the tissue with the back of a syringe in sterile medium and passing the resulting mixture through a sterile wire mesh. Bone marrow cells were flushed out of the femoral cavity with PBS. Peritoneal cells were obtained by lavage with 2-4 ml PBS.

Flow Cytometric Analysis and Antibodies Used for Staining. Two-

and three-color flow cytometric analyses were performed as previously described (16). Briefly, 0.3-1.0 × 10^6 cells were stained with optimal concentration of antibody for 45 min at 4°C and then washed three times. For indirect staining, second antibodies or streptavidin conjugates were incubated for 45 min at 4°C. After washing, cells were lysed with Coulter Lyse and fixed with Coulter Fix (Coulter Corporation, Hialeah, FL) before washing three times. All steps were performed in Coulter PBS (Coulter Corporation). Analysis of fixed cells was performed over the next 2 d using a FACScan® (Becton Dickinson Immunocytometry Systems, Mountain View, CA). We purchased from Becton Dickinson Immunocytometry Systems the following antibodies which are specific for human cell surface markers: CD3 (FITC and PE), CD4 (FITC and PE), CD5 (FITC), CD8 (PE), CD19 (PE), CD20 (PE), CD25 (PE), CD45 (FITC), CD45RA (FITC), anti-Transferrin Receptor (FITC), HLA-DR (FITC, PE), TCR α/β (FITC), TCR γ/δ (FITC), Leu8 (FITC), and CD1a-purified antibody. All mouse isotype controls were also obtained from Becton Dickinson Immunocytometry Systems. The monoclonal specific for CD45RO (FITC) was purchased from Dako Corporation (Santa Barbara, CA). Biotinylated anti-H-2K^d was purchased from Pharmingen (San Diego, CA). PE-conjugated streptavidin came from Caltag Laboratories (South San Francisco, CA). All these antibodies exhibited species specificity when tested on mouse and human cells. To exclude the possibility of murine cells staining nonspecifically for human markers (12), we used two-color FACS® analysis. Human cells were stained for the leukocyte common antigen CD45 and murine cells were stained for the murine MHC class I molecule H-2K^d. The antibodies did not cross-react between cells of the two species (Fig. 3 A), and we could clearly distinguish cells of human (CD45$^+$, H-2K^{d-}), and murine (CD45$^-$, H-2K^{d+}) origin in engrafted mice. Cell mixing experiments revealed the detection limit of 1 human cell in 1,000 murine cells (data not shown).

Cell Cycle Analysis. Cell cycle analysis was performed as described (16, 17). Cells were first stained with FITC-labeled anti-CD3 or anti-CD45 antibodies (see above), fixed in 2% paraformaldehyde solution, and the membranes were permeabilized with 0.2% Tween 20 (Sigma Chemical Co., St. Louis, MO) in PBS. DNA was stained by incubating 10^6 cells in 1 ml of PBS containing 10 μg/ml of propidium iodide (Calbiochem Corp., La Jolla, CA) and 11.25 Kunitz Units of RNase (Sigma Chemical Co.) for 30 min. The samples were analyzed on a FACScan® flow cytometer (see above).

T Cell Proliferation Assay with Cells Freshly Prepared from Chimera Organs. Single-cell suspensions were prepared from the lungs, liver, and spleen of individual mice as described above. Cells were pooled and the relative number of human (CD45$^+$) vs. murine (H-2K^d) cells was determined by FACS® analysis. The cell suspension was plated into 96-well flat-bottomed plates (Corning Glass Works, Corning, NY) titrating from 10^6 to 10^4 cells per well. The number of human cells per well was calculated by subjecting the cell suspension to FACS® analysis and determining the murine/human cell ratio (usually 20-50%). Human APC were added. The APCs were derived from the same human donor with whose cells the hu-PBL-SCID mouse had been reconstituted. The APC comprised PBMC that had been depleted of T cells by rosetting with sheep erythrocytes (18) and then irradiated (3,000 rad). Anti-CD3 antibody (Orthoclone OKT3; Ortho Pharmaceutical Corp., Raritan, NJ) was used at 1 μg/ml final concentration, which has been established as maximally stimulatory for human PBMC and T cell lines. rIL-2 (a gift of Sandoz Ltd., Basel, Switzerland) was used at 10 ng/ml final concentration. The culture medium consisted of DMEM supplemented with 10% FCS, 1% glutamine, 1% nonessential amino

acids, and 3 × 10⁻⁵ M 2-ME. After 5 d of culture, 1 μCi of [³H]thymidine was added to each well. 18 h later, the plates were harvested and ³H incorporation was counted by liquid scintillation. The results are expressed as incorporation of radiolabel by the cells (arithmetic mean ± SD).

Generation of Human T Cell Lines from hu-PBL-SCID Mice. Primary cultures from hu-PBL-SCID mice contained (*a*) a single cell suspension from the organs of the chimeras at 4 × 10⁶ total cells/ml, (*b*) irradiated, syngeneic, human PBMC at 2 × 10⁶ cells/ml; (*c*) anti-CD3 mAb at 1 μg/ml; and (*d*) rIL-2 at 10 ng/ml final concentration. The culture was performed in 24-well plates (Corning Glass Works) in 2 ml of DMEM-FCS medium (see above). 7 d later, viable cells were readjusted to 3 × 10⁵ cells/ml and cultured in 24-well plates in DMEM-FCS medium containing rIL-2 (10 ng/ml). At this point no feeder cells or CD3 mAb were added. On day 15 the lines were readjusted to 2 × 10⁵ cells/ml and were restimulated with irradiated, syngeneic, human APC (PBMC, 2 × 10⁶/ml), CD3 mAb (1 μg/ml), and rIL-2 (10 ng/ml) in 24-well plates in 2 ml DMEM-FCS medium per well. Thereafter, the cells were fed weekly with fresh DMEM-FCS medium containing rIL-2 (10 ng/ml) and restimulated with syngeneic, human APC and Ag (CD3 mAb) once a month according to the protocol previously described (19).

T Cell Proliferation Assay with Chimera-derived Human T Cell Lines. T cells lines were not tested within 2 wk of restimulation with CD3 antibody and APC. Thereafter, the assays, were performed with 3 × 10⁴ cells of the chimera-derived cell line (<97% CD45⁺, CD3⁺ by day 15) and 10⁵ human, autologous, T cell–depleted (E-rosetting), irradiated (3,000 rad) PBMC as APC. The proliferative potential of the line was assayed by polyclonal stimulation with CD3 mAb (1 μg/ml) or PHA (Sigma Chemical Co.) at 3 μg/ml which formally has been established as the optimal concentration for induction of blastogenesis. The antisecondary host response was tested by adding irradiated (3,000 rad), SCID-splenic stimulator cells at 2 × 10⁵ cells/well. The cells were cultured in 200 ml complete DMEM in 96-well flat-bottomed plates (Corning Glass Works). After 3 d of culture, 1 μCi [³H]thymidine was added to each well. The plates were harvested 18 h later, and [³H]thymidine incorporation was assayed by liquid scintillation counting as described above.

Gene Amplification (PCR) for EBV Genome. DNA was prepared for PCR according to Higuchi (14). Cells were washed twice with PBS and resuspended at 6 × 10⁶ cells/ml in PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2.5 mM MgCl₂ 0.1 mg/ml gelatin, 0.45% NP-40, 0.45% Tween 20). This mixture was incubated with 10 μg/ml of proteinase K for 1 h at 60°C and then at 95°C for 10 min. The samples were then frozen at −70°C until used. Nucleotides 1396–1520 of the EBV BamW region were amplified with primers TC-60: 5'dCCAGAGGTAAGTGGACTT and TC-61: 5'dGACCGGTGCCTTCTTAGG developed by Fox et al. (13) and synthesized by Genosys, Inc. (Houston, TX). Each PCR reaction mixture contained 1 μg PCR-prepared DNA, 300 pM TC-60 and TC-61 primers, 100 pM dNTP pools and PCR reaction buffer (10 mM Tris-HCl at pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin (No. G2500; Sigma Chemical Co.). Each sample was capped with 50 μl of light mineral oil, then denatured (94°C, 10 min) and cooled (55°C, 30 min). 2.5 U of *Thermus aquaticus* (*Taq*) DNA polymerase (Perkin-Elmer/Cetus, Norwalk, CT) was added to each sample bringing the final reaction volume to 100 μl. The samples were subjected to 40 successive cycles of denaturation (91°C, 1 min), primer annealing (72°C, 1 min) and primer extension (65°C, 2 min). All reactions were performed in a DNA Thermal Cycler (Perkin-Elmer/Cetus). For Southern blot analysis of the PCR products, 10 μl of each amplified DNA sample was run on a 1.6% agarose gel and transferred to a Zeta-Probe blotting membrane (Bio-Rad Laboratories, Richmond, CA). This was then hybridized with 20 pg of an ³²P end-labeled oligonucleotide sequence (TC62 = dTTCTGCTAAGCCAACC [13]) internal to the primers; then rinsed twice in 2 × SSPE (1 × SSPE: 0.18 M NaCl, 10 mM NaH₂PO₄, 1 mM EDTA, pH 7.4) and 0.05% SDS for 15 min at room temperature.

Results

The persistence of mature human leukocytes in SCID mice, as well as the establishment of human hematopoiesis in the mice after transfer of human lymphoid cells, depend on poorly understood cooperative effects between the different cell types of the graft as well as host factors. Initially, we tested cells from various human lymphoid organs to determine which cells would provide the most favorable reconstitution of a human immune system in the xenogeneic recipient. We injected unmanipulated SCID mice intraperitoneally with single cell suspensions from PBL, cord blood, bone marrow, or thymus. The success of engraftment was monitored by FACS® analysis of the organs of grafted mice for the presence of various human leukocyte populations, and measuring human Ig in the serum of the recipient mice.

Human Leukocytes Can Be Readily Detected in SCID Mice Injected with PBMC and Cord Blood, but Not in Mice Injected with Bone Marrow or Thymocytes. The organs of grafted SCID mice were analyzed for human leukocytes at different times after transplantation. Representative data selected from 59 mice tested individually are shown in Table 1. In the first 20 d after intraperitoneal inoculation of PBL, human leukocytes could be detected at the site of injection, in the peritoneal cavity, but not in other organs of the mice. Initially, the number of human cells dropped dramatically: from 10.0 × 10⁶ injected PBL < 0.01 × 10⁶ cells (1:1,000) were recovered from the peritoneal cavity 7 d later. At about day 30 after reconstitution with PBL or cord blood, human leukocytes became detectable in various organs of the mice (Table 1). The number of human cells increased steadily within the first 3 mo after injection to eventually constitute up to 50% of all mononuclear cells recovered from the organs of chimeras. Subsequently, the number of human cells declined so that by day 200, generally no human cells could be detected. The disappearance of the human cells was associated with the decline of human serum Ig levels in the mouse (see below). We could not detect human leukocytes in the mice injected with bone marrow and thymocytes early or late after grafting (Table 1).

Human Ig's Can Be Detected in Sera of SCID Mice Injected with PBMC, Cord Blood and, to a Lesser Extent, Bone Marrow. When 10–15 million PBMC from five different donors were injected, 15 of 34 mice (44%) developed human serum Ig levels >700 μg/ml, which was indicative of the presence of human cells (see below). After injection of 20 million PBMC from six different donors, 22 of 40 mice (55%) became positive for human Ig's. Representative data from these experiments are shown in Fig. 1 A. Since injection of higher numbers of PBMC did not yield better results but led to in-

creased mortality of recipient mice (data not shown), we used 20 million PBMC for reconstitution in subsequent experiments. We did not observe an influence of the donor's EBV status on the reconstitution. We injected 57 mice with EBV⁺ PBL and only two (3.5%) developed a B cell lymphoma as detected by PCR.

After injection of 20 million cord blood mononuclear cells from five different donors, 5 of 26 mice (19%) exhibited human Ig's >700 µg/ml in their serum over an observation period of 100 d. Five of five mice injected with 35 million cord blood cells were found to be reconstituted by this criterion (Fig. 1, B). The time course, as well as the peak Ig concentration, was comparable in cord blood- and PBMC-reconstituted mice.

IgG was the predominant human Ig class in the chimeras. Essentially identical results were obtained for IgM (data not shown).

We inoculated bone marrow cells from four different donors at 10–30 million mononuclear cells per recipient mouse. 4 of 11 injected animals developed detectable human Ig's, but the serum concentration was rather low (Fig. 1, C). After injection of human thymocytes, which contain up to 3% B cells (21), no human Ig was detected (Fig. 1 D).

Correlation Between Human Leukocytes and Human Ig Levels in the Chimeras. As stated before, PBL of some human donors consistently failed to induce production of human Ig in SCID mice. Even "good" donors gave rise to high level Ig produc-

Table 1. *Detection of human CD45⁺ leukocytes in SCID mice*

					Percent CD45⁺ leukocytes detected in organs of chimeras						
Human cells injected			Tested on day	Human Ig*	Intraperitoneal fluid	Lung	Liver	Spleen	Thymus	Bone marrow	Blood
Type	Donor	No.									
		× 10⁶		µg/ml							
PBMC	A	10	7	0	10	<1	<1	0	0	0	0
	F	20	30	35	<1	0	0	0	0	0	0
	A	20	34	3,275	67	24	23	42	10	<1	7
	A	10	35	2,848	ND	22	20	32	ND	1	4
	D	20	35	1,413	49	21	18	48	62	1	2
	F	20	63	14,825	26	38	21	57	19	9	ND
	B	10	74	790	ND	54	35	12	22	1	3
	A	20	97	1,408	7	9	10	34	0	2	0
	F	20	115	3,868	24	18	7	3	9	1	14
	A	10	164	254	0	3	2	3	<1	<1	0
Cord blood	N	35	33	1,700	36	4	1	<1	<1	0	<1
	L	20	45	1,938	22	2	0	0	48	0	0
	N	35	54	3,000	64	23	11	43	10	1	2
	L	20	121	8,108	<1	6	6	8	17	2	2
	L	20	132	236	2	0	0	0	0	0	0
Thymocytes	T	100	53	0	0	0	0	0	0	0	0
	S	20	70	0	0	0	0	0	0	0	0
	S	20	70	0	0	0	0	0	0	0	0
	T	100	122	0	0	0	0	0	0	0	0
Bone marrow	J	30	55	0	0	0	0	0	0	0	0
	K	10	81	0	0	0	0	0	0	0	0
	J	30	153	258	0	0	0	0	0	0	0
	J	30	153	163	0	0	0	0	0	0	0

Mice were injected intraperitoneally with human PBMC, cord blood mononuclear cells, thymocytes or bone marrow cells. Donors are ide tified by capital letters and the number of cells grafted is shown. On the days indicated, recipient mice were killed, and single cell suspensions f the organs were studied by two-color FACS® analysis. Human leukocytes were stained with anti-CD45-FITC and murine cells were identified by anti-H-2K^d-PE. The frequency of human leukocytes within an organ is expressed as percent CD45⁺ cells [%CD45 = 100 × (CD45⁺, H-2K^d- cells/CD45⁻, H-2K^d+, ungated)].
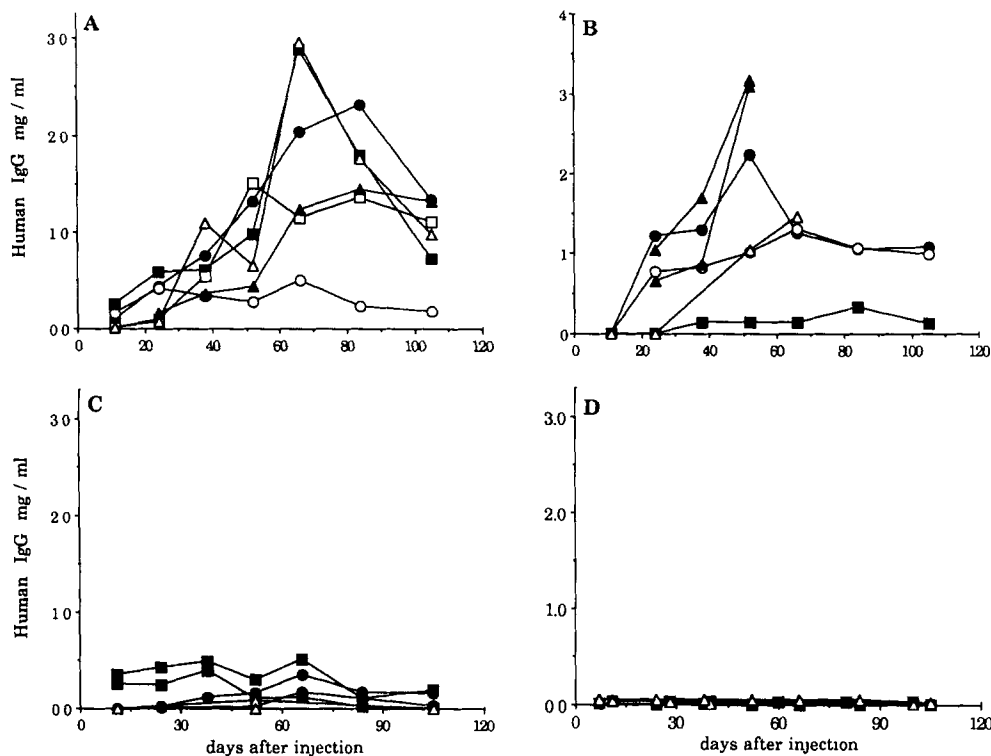* Human serum Ig levels in the mice on the day of FACS® analysis.

Figure 1. Detection of human Ig's in sera of SCID mice transplanted with human lymphoid tissues. Human cells were injected in single cell suspension intraperitoneally. Recipient mice were bled biweekly. The concentration of human antibodies in the serum was tested by ELISA. (*A*) PBMC-injected mice after inoculation of 2 × 10⁷ cells. Individual mice, each grafted with cells of a different donor are shown. The data are representative for a total of 30 mice injected with PBMC of six donors. Antibody production was donor dependent. PBL of some donors consistently failed to repopulate the mice in repeated experiments. (*B*) Cord blood–injected mice, after inoculation of 2–3.5 × 10⁷ cells. Individual mice are shown. Symbols denote different donors. The data are representative of 26 mice injected with five donors. (*C*) Bone marrow–injected mice. 1–2 × 10⁷ (*closed* symbols) and 3 × 10⁷ (*open* symbols) from four donors (■, ▲, ●, △) were injected. None out of 11 injected mice developed human IgG >700 µg/ml in their serum. (*D*) Thymocyte-injected mice. In two experiments, 2 × 10⁷ cells of one donor (*closed* symbols, eight mice) and 10 × 10⁷ of another donor (*open* symbols, three mice) were injected.

tion in only 75% of the injected mice. In mice that did not develop human Ig levels >700 µg/ml, no human cells could be detected by FACS® analysis, irrespective of whether the mice were tested early or late after transplantation (13 of 13 mice tested). Conversely, animals with human Ig >700 µg/ml had detectable numbers of human leukocytes (36 of 39 mice) as well. Furthermore, the decline of human Ig in reconstituted mice was associated with human cells dropping beyond the detection limit in chimeras (seven of seven mice). These data are summarized in Fig. 2 *A*. Regression analysis for PBL-injected mice (see Fig. 2 *A* legend) revealed a highly significant correlation between human IgG levels and the detectability of human leukocytes in the lungs. Virtually identical correlation was found for the liver. The analysis indicates that to achieve a 95% probability of finding a non-zero number of CD45⁺ cells in these organs, animals with human IgG levels >700 µg/ml have to be selected. The correlation was weaker in the spleen and, due to fewer data points, in human cord blood injected mice. Occasionally, the human cells were abundant in one of the chimera's organs (lung, liver, or spleen), but low in number in the other organs (Fig. 2 *B*).

Human Leukocytes Preferentially Reside in the Lungs, Liver, and Spleen of the Chimeras. Few Human Cells Repopulate Mouse Thymus, Bone Marrow, and Lymph Nodes. After injection of PBL or cord blood cells, human leukocytes were detected in all organs of the chimeras that were accessible to FACS® analysis (Table 1). However, the human cells were preferentially found in the lungs, liver, and spleen (Fig. 2 *B*). Less than 4% of the cells in the bone marrow were CD45⁺. Occasionally, a mouse thymus contained up to 60% human cells, but this is deceptive as the thymus of the chimeras, like the thymus of SCID mice, is rudimentary and consists of <3 × 10⁶ cells (normal, age-matched murine thymuses have 100–300 × 10⁶ cells). Also, the lymph nodes of the chimeras were hypoplastic, typically being too small to detect visually. Thus, based on absolute numbers, >96% of the human leukocytes recovered from the chimeras were found in the lungs, liver, and spleen.

The Predominant Human Cell Type in the Chimeras Are Mature T Lymphocytes that Express Activation Markers. In all organs of the chimeras, human T cells prevailed (Table 2). This dominance of T cells was seen in all chimeras (30 mice), irrespective of whether PBL or cord blood cells were used for reconstitution, and irrespective of the time point at which the mice were analyzed after transplantation. Human B cells (CD19⁺ and/or CD20⁺) were detected in 7 of 23 PBL-reconstituted and five of seven cord blood–injected mice. The number and tissue distribution of B cells showed a high de-
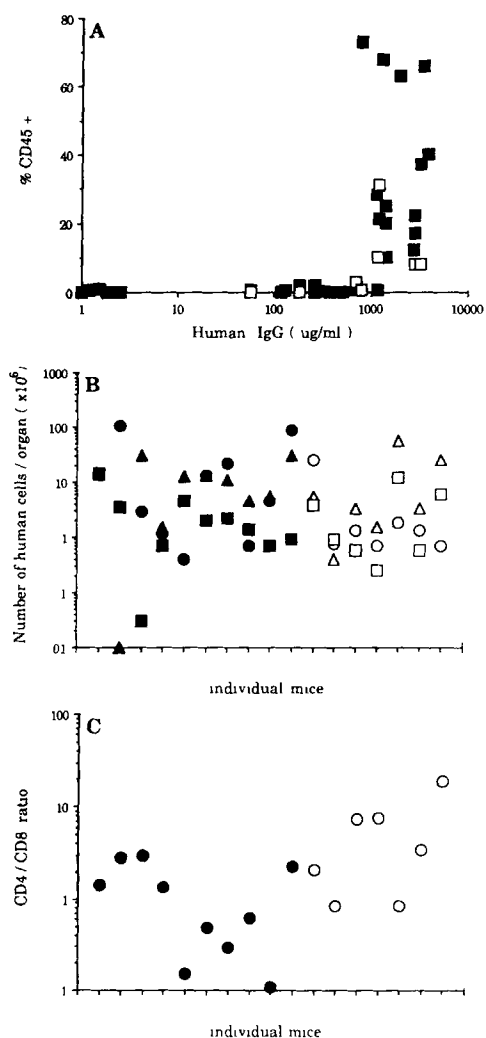
Figure 2. Detection of human leukocytes in hu-PBL-SCID mice. In all panels *filled* symbols represent PBL-injected mice, *open* symbols represent cord blood-injected mice. Symbols used for organs of the chimeras are lungs (*squares*), livers (*triangles*), and spleens (*circles*). (*A*) Correlation between human serum Ig levels and the detectability of human leukocytes. The percentage of CD45+, H-2K^{d-} cells in lungs of PBL- or cord blood–injected chimeras was defined by FACS® analysis (see Table 1, legend) and plotted against the concentration of human IgG in the serum for individual mice (measured by ELISA). All mice tested during our study are included, irrespective of the donor or time point after grafting (7–164 d after injection, $n = 50$). Statistical analysis of the correlation between human IgG levels and human leukocytes in the lungs of PBL-injected mice: $r^2 = 0.363$, $t = 4.45$, $p < 0.001$, $F = 19.8$. Similar correlation was seen for the liver and spleen of PBL-injected mice: $r^2 = 0.363$, $t = 4.45$, $p < 0.001$, $F = 19.8$ and $r^2 = 0.170$, $t = 2.8$, $p < 0.009$, $F = 7.76$, respectively (not plotted). The correlation for lungs of cord blood-grafted mice ($n = 8$): $r^2 = 0.397$, $t = 4.45$, $p < 0.056$, $F = 5.62$. The data for liver and spleen of cord blood-injected mice: $r^2 = 0.123$, $t = 1.5$, $p < 0.18$, $F = 2.24$ and $r^2 = 0.123$, $t = 1.0$, $p < 0.35$, $F = 0.98$. Note that when few human leukocytes were present in the lungs of Ig+ mice, CD45+ cells frequently were abundant in the liver or spleen (*B*). (*B*) Absolute numbers of CD45+ cells recovered from lungs, livers, and spleens of individual chimeras. Representative data are shown for chimeras (human IgG > 700 µg/ml) that had been tested 35–80 d after transplantation. For calculation of absolute numbers, the percentage of CD45+, H-2K^{d-} cells within the ungated single-cell suspension of an organ was determined by two-color FACS® analysis. With human cell counts <<1 million per organ of chimeras, data for thymus, bone marrow, blood and intraperitoneal fluid

gree of inter-individual variation. In some chimeras, particularly in cord blood–injected mice, the thymus and the spleen contained up to 77.7% B lymphocytes (Table 2). We tested whether the occasional occurrence of a high percentage of B cells in the chimeras was related to EBV tumors: PCR could not detect the EBV genome in the cells in question. The presence of human B cells in the thymus of the chimeras might reflect a physiologic outcome of the transplantation since mature B cells constitute 3% of the thymocytes in adult, healthy mice (20), as well as in humans (21).

Fig. 3 shows results of FACS® analysis obtained with the thymus of an individual PBL-reconstituted mouse. The data are representative of all organs (spleen, lungs, liver, bone marrow, and lymph nodes) of 36 chimeras tested individually, between days 35 and 164 after grafting. As shown in Fig. 3 *A*, we could clearly distinguish between cells of murine (H-2K^{d+}, CD45$^-$) and cells of human (H-2K^{d-}, CD45$^+$) origin. Human cells constituted 20% of all cells in the thymus of this mouse. Furthermore, it is clear that all human cells in the chimera were leukocytes, since we did not detect an H-2K^{d-}, CD45$^-$ cell population. In this mouse, basically all CD45$^+$ cells represented T cells that stained with CD3 (Fig. 3 *B*) and with CD2 (data not shown). Less than 1% B cells were detected (Fig. 3 *C*). In other chimeras in which B cells are present (Table 2), B and T cells accounted for >99% of the CD45$^+$ cells. Thus, T and B cells were the only human leukocytes we detected in the chimeras. The CD3$^+$ T cells expressed CD4 or CD8 in a mutually exclusive manner (Fig. 3, *D*–*F*). In general, the ratio of CD4$^+$ to CD8$^+$ cells showed a high degree of interindividual variability in hu-PBL-SCID mice with dominance of either cell type (Fig. 2 *C*; the normal range for CD4/CD8 ratio in adult PBL is 0.93–4.5). Double-positive (CD4$^+$, CD8$^+$) T cells were occasionally seen in low numbers (<4% of CD45$^+$ cells) in various organs of the chimeras. The double positive cells also expressed the marker CD45RO (Fig. 3 *J*).

All T cells in the chimera expressed the α/β TCR and no γ/δ cells were detected (Fig. 3, *G* and *H*). All T cells were positive for CD45RO, the low molecular weight isoform of CD45, and were negative for CD45RA (Fig. 3, *I* and *J*). Also, most T cells were HLA-DR$^+$ (Fig. 3 *K*), and did not express the homing receptor Leu8 (Fig. 3 *L*). Essentially identical results were obtained after staining organs of cord blood–reconstituted SCID mice (Fig. 4): T cells prevailed that had the phenotype of mature (CD4$^+$ or CD8$^+$), memory-type (CD45RO$^+$, CD45RA$^-$), and recently activated (HLA-DR$^+$, Leu-8$^-$) T lymphocytes.

are not represented. (*C*) CD4/CD8 ratio in the chimeras. Mice that have been tested 30–70 d after injection of PBL or cord blood are shown. The percentage of CD45+, CD4+, and CD45+, CD8+ cells within the CD45+ population was determined by two-color FACS® analysis and the CD4/CD8 quotient was calculated. The ratio was comparable within different organs of individual mice, showed donor dependence and did not appear to change with time (not shown).

Table 2. *Detection of Human T and B Lymphocytes in PBMC – or Cord Blood–grafted Mice*

	Relative percent CD45⁺ cells			
	PBMC-reconstituted mice		Cord blood–reconstituted mice	
SCID organ	T (CD3⁺)	B (CD19⁺,CD20⁺)	T (CD3⁺)	B (CD19⁺,CD20⁺)
Blood	>99 ± 0.0	<0.0 ± 0.0	>99 ± 0.0	<0.0 ± 0.0
Bone marrow	>99 ± 0.0	<0.0 ± 0.0	>99 ± 0.0	<0.0 ± 0.0
Thymus	97.1 ± 9.5	2.9 ± 9.5	82.3 ± 28.6	17.6 ± 28.6*
Peritoneal fluid	97.7 ± 5.6	2.2 ± 5.6	99.3 ± 0.9	0.6 ± 0.9
Spleen	98.7 ± 4.7	1.2 ± 4.7	88.1 ± 22.5	11.8 ± 22.5
Liver	99.2 ± 1.9	0.7 ± 1.9	97.4 ± 3.9	2.5 ± 4.0
Lungs	99.6 ± 0.8	0.4 ± 0.8	89.1 ± 14.8	10.8 ± 14.8

Data obtained from analysis of 23 PBMC- and seven cord blood–grafted SCID mice are summarized. Mice were killed between days 30 and 160 after grafting, and the indicated organs were studied by two-color FACS® analysis. The antibody combination CD45-FITC and CD19/CD20-PE was used to detected B cells. T cells were stained with CD45-FITC and CD3-PE (original data are plotted in Figs. 3 and 4). The high standard deviations are due to the occasional occurrence of B cells in high numbers, i.e., in the thymus of cord blood–injected mice.
*77.7%, 2.3%, 43.1%, 0%, 0%, 0%, 0% were B cells.

Human T Cells Proliferate in the Chimeras. Proliferation of human T cells in the mice was suggested by the fact that the number of T cells recovered increased with time (Table 1). To define the mitotic activity of the human T cells in vivo in the chimeras, we analyzed cells freshly isolated from the chimeras. Propidium iodide was used to stain DNA, while cells were labeled with the anti–CD3 antibody. This allowed us to determine the DNA content of the human T cells by two-color FACS® analysis. As shown in Fig. 5 *A*, 18% of the CD3⁺ cells in a chimera were in the process of DNA replication (stages S, G2, and M of the cell cycle). This represents a rather rapid mitotic activity since the continuously growing human immature T leukemia cell line CEM exhibits 44% of its cells in the process of DNA replication when tested under the same conditions (Fig. 5 *C*). As a negative control, we used otherwise untreated, freshly isolated human PBL that showed no evidence of DNA replication (Fig. 5 *B*).

Human T Cells in the Chimera Are in a State of Anergy from which They Recover after Long-Term Culture In Vitro Displaying Xenoreactivity. We next tested whether the human T cells in the chimeras were functional. Cells were isolated from the chimeras' organs and tested in vitro for proliferation to stimulation via the TCR. The T cells did not respond to triggering with the anti–CD3 antibody in the presence (or absence) of syngeneic human APC (T cell–depleted, irradiated PBL) (Table 3). We also did not detect IL-2 in the supernatant after stimulation. The complete lack of a proliferative or IL-2 response was also seen after stimulation with the T cell mitogen PHA (data not shown). However, the freshly isolated human T cells from the chimeras did proliferate in the presence of exogenous IL-2 (Table 3).

We established 11 long-term, human T cell lines from hu-PBL-SCID by culturing freshly isolated cells with anti-CD3 and IL-2 in the presence of syngeneic, human feeder cells (see Materials and Methods). This protocol was chosen to assure polyclonal expansion of the chimera-derived human T cells. As determined by FACS® analysis, by day 15 of the culture, the cell lines consisted <97% of human T cells (data not shown). At this time, all the T cell lines were still refractory to stimulation by the CD3 antibody (Table 3). When seven lines were tested on day 40, three lines responded with proliferation to CD3 stimulation while four lines were still unresponsive. After 2 mo of culture, all cell lines displayed proliferative responses to CD3 (Table 3) and this functional state remained stable over the next 6 mo.

After the human T cell lines established from the chimeras had become responsive to stimulation via CD3, we tested whether these lines exhibited xenoreactivity to murine antigens. The T cells were cultured with SCID spleen stimulator cells in the presence of human APC (T cell–depleted, syngeneic, irradiated PBMC). As shown in Table 3, the majority of T cell lines proliferated specifically to SCID stimulator cells (9 of 11 lines tested in total). No proliferation was elicited by C57.BL/6 splenic stimulator cells (data not shown). The xenoreactivity of the T cell lines was maintained over the entire period of subsequent culture (180 d) in which the lines were expanded polyclonally by anti-CD3 and IL-2 stimulation. The xenoresponse was dependent on the presence of syngeneic human APC (data not shown). The two lines that did not proliferate to SCID stimulator cells consisted predominantly of CD8⁺ lymphocytes (92 and 97%), whereas in the responding lines CD4⁺ cells dominated (>60%).
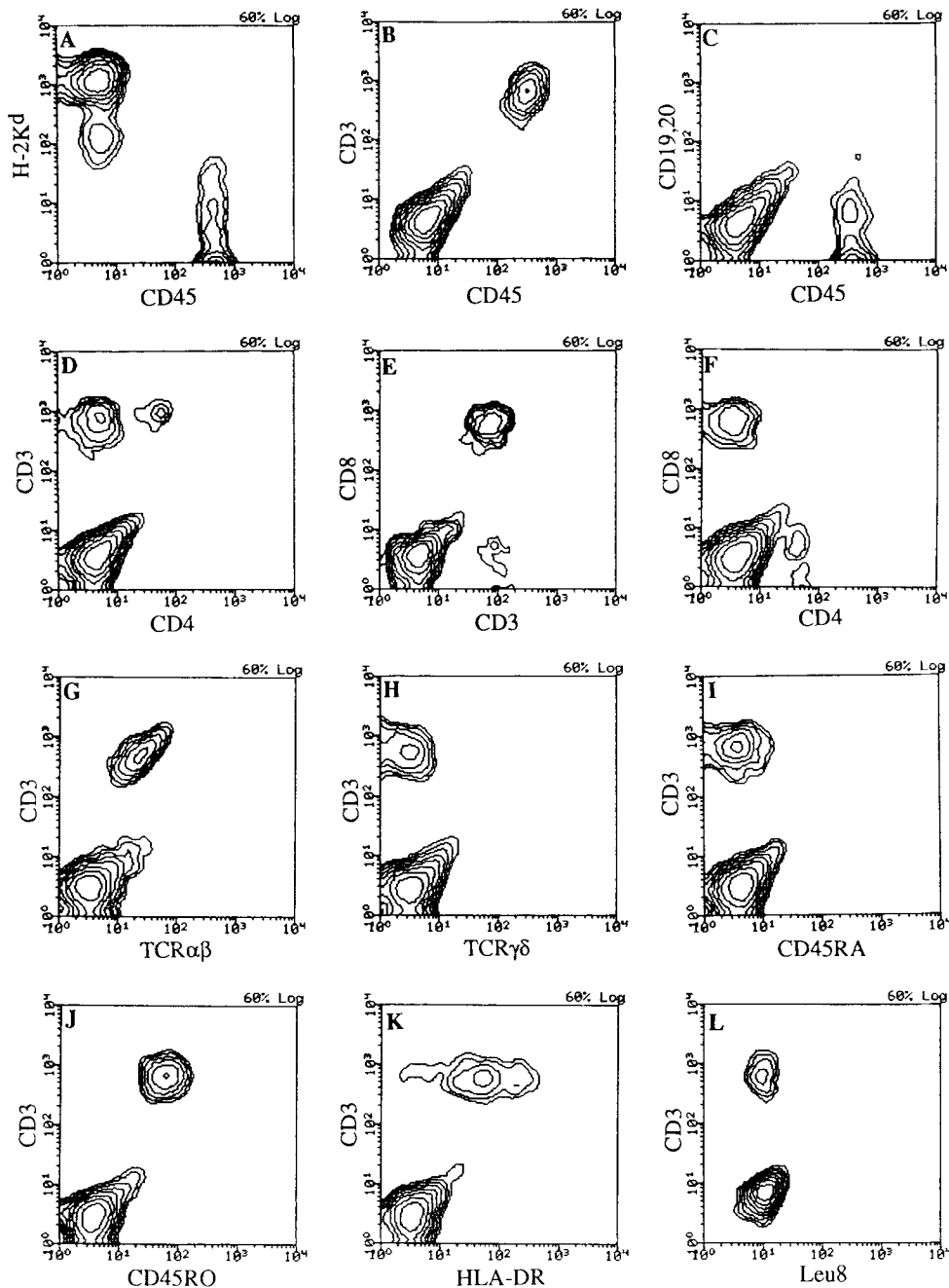
Figure 3. Phenotypic characterization of human leukocytes in hu-PBL-SCID mice. Two-color FACS® analysis of the thymus of a chimera 50 d after grafting with 20 × 10⁶ PBMC. In all diagrams, the antibodies for fluorescence 1 (x-axis) were FITC-labeled and the antibodies for fluorescence 2 (y = axis) were PE labeled. Identical results were obtained by analyzing the liver, spleen, and lungs of the same chimera (not shown).

Discussion

Establishment of a human immune system after transfer of human lymphoid cells into SCID mice may be achieved in four different ways: I. Human stem cells may home to murine primary lymphoid tissues (bone marrow and thymus) or to tissues that can support hematopoiesis (liver and spleen) where they give rise to human lymphoid lineages. Resulting mature lymphocytes may repopulate the secondary lymphoid tissues to eventually form a functional immune system. II. Mature human lymphocytes may persist in the mouse (22). In this case the life span and function of individual human

leukocyte lineages after transplantation will define the human immune system in the chimera. For example, it is conceivable that certain long-lived lymphocytes (22) persist in the secondary host while short-lived lymphocytes (23) would die off rather rapidly. III. It is possible that mechanisms that regulate leukocyte homeostasis drive the repopulation of the mice (24, 25). In this case, certain self-replenishing leukocyte lineages (26), which have the capacity to proliferate in the absence of antigenic stimulation, may repopulate the mice. IV. It is possible that the human cells do react to murine antigens
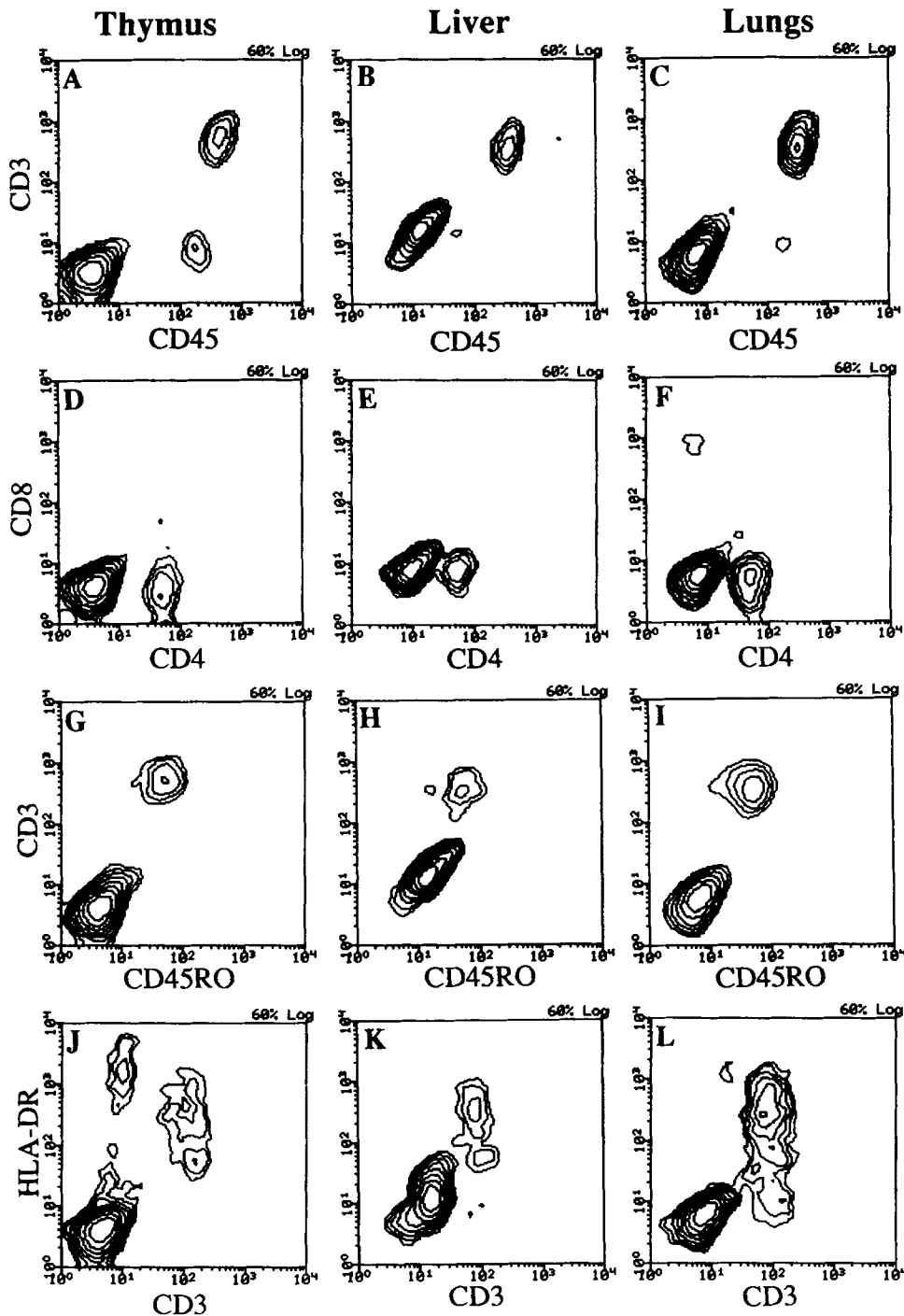
Figure 4. Phenotypic characterization of the human leukocytes in cord blood-reconstituted SCID mice. Two-color FACS® analysis with the thymus, liver, and lungs of a chimera 54 d after grafting. Antibodies for fluorescence 1 (x-axis) were FITC, and antibodies for fluorescence 2 (y-axis) were PE-labeled. The spleen of the same chimera revealed basically identical results (not shown). These data are representative for seven mice reconstituted with cord blood from five donors and tested between days 33 and 121 after grafting.

that may lead to preferential expansion and repopulation with xenoreactive lymphocytes, although in our hands and in the hand of others (27) there is no evidence for acute GVHD in the chimeras. V. A combination of mechanisms I–IV is also conceivable.

Our studies did not provide evidence for human lymphopoiesis (possibility I): the double-positive T cell phenotype (CD4+,CD8+), which characterizes the major population of immature thymocytes, was seen rarely and in low numbers in the chimeras at any time point (<4% of the human T cells, Figs. 3 and 4). Mature, single-positive T cells prevailed from day 30 after transplantation, the earliest time point when human leukocytes could be detected in organs of the mice, through day 160, after which the human leukocytes usually were no longer detectable. When seen, CD4/CD8 double-positive T cells were present not only in primary lymphatic tissues (thymus, and possibly liver and spleen), but also in the lungs. All double-positive T cells were CD3+, CD1−
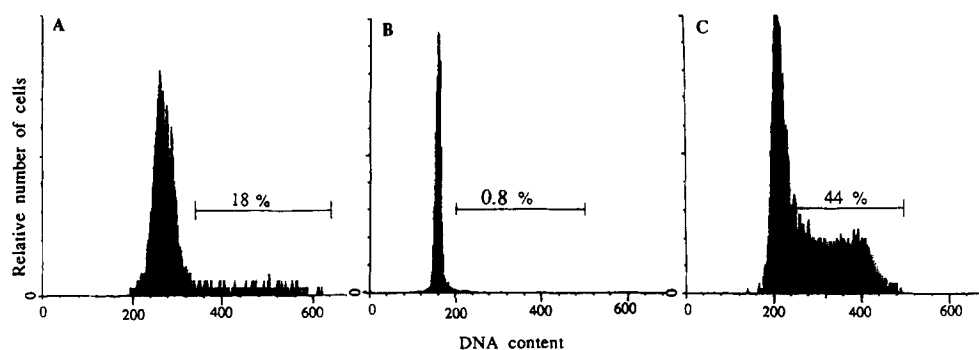
Figure 5. Cell cycle analysis of T cells in hu-PBL-SCID mice. (*A*) Human T cells in the liver of a chimera 50 d after grafting with 2 × 10⁷ PBMC. The single-cell suspension was labeled with anti-CD3-FITC and the DNA was stained with propidium iodide. The samples were studied by two-color FACS® analysis. The DNA content is plotted versus the relative number of CD3⁺ cells. This is a representative example of seven experiments on SCID mice that had been grafted with PBMC and cord blood 41 to 154 d before. (*B*) Freshly prepared, unstimulated PBMC of the same donor (anti-CD3 stained), and (*C*) leukemia cell line CEM are shown as controls. Since CEM does not express TCR on the surface, in (*C*) anti-CD45-FITC was used to label the cells. The percentage of cells in phases S, G2, and M of the cell cycle is indicated.

CD45RO⁺. This phenotype might be consistent with a lineage of immature T cells that is committed to intrathymic death (28, 29). Alternatively, the CD45RO⁺, double-positive cells might represent mature, activated lymphocytes (30, 31) that can coexpress CD4 and CD8 (32–34). Also, the double-positive cells in the thymus of the chimera could represent mature, activated cells that preferentially reenter this organ (35). Since we did not detect (immature or mature) human T cells in SCID mice after grafting with stem cell–rich

human thymus or bone marrow (Table 1), we suggest that the rare CD4⁺, CD8⁺ population in the chimeras represents activated, mature T cells.

Why human lymphopoiesis does not readily occur in SCID mice after transplantation of human stem cells is unclear. It is possible that the human stem cells are rejected by NK cells (36) in the unmanipulated recipients. Alternatively, the murine hematopoietic stroma might not provide the appropriate microenvironment (cytokines and adhesion molecules) for

Table 3. *Proliferative Responses of Chimera-derived Human T Cells*

	Assay conditions				Exp. 1		Exp. 2	
Day tested	T	APC	Ag	IL-2	A no. 1(E)	A no. 2(C)	A no. 3(B)	A no. 4 (E)
0	+	−	−	−	3.4 ± 1.1	3.8 ± 0.4	5.9 ± 1.3	2.4 ± 0.1
	+	+	−	−	4.2 ± 1.1	4.1 ± 0.9	7.6 ± 0.3	4.9 ± 0.5
	+	+	αCD3	−	3.7 ± 0.9	3.1 ± 0.9	10.1 ± 0.8	3.5 ± 0.5
	+	+	αCD3	+	43.6 ± 4.2	26.8 ± 5.7	37.2 ± 1.5	120.3 ± 2.3
	+	+	−	+	27.5 ± 1.2	20.1 ± 5.4	26.3 ± 4.1	90.6 ± 1.2
15	+	+	−	−	7.8 ± 0.6	10.6 ± 0.4	0.4 ± 0.1	0.7 ± 0.1
	+	+	αCD3	−	18.5 ± 4.1	11.8 ± 0.2	1.2 ± 0.5	1.5 ± 0.1
40	+	+	−	−	11.6 ± 3.2	3.7 ± 1.8	2.9 ± 0.1	53.9 ± 0.3
	+	+	αCD3	−	138.8 ± 7.3	5.5 ± 1.1	3.5 ± 0.1	173.4 ± 13.6
68	+	+	−	−	0.7 ± 0.03	0.9 ± 0.01	1.4 ± 0.1	2.6 ± 0.3
	+	+	αCD3	−	112.1 ± 9.1	158.2 ± 6.3	16.3 ± 2.3	198.2 ± 2.7
	+	+	SCID	−	19.7 ± 3.9	1.3 ± 0.1	22.1 ± 0.9	27.2 ± 2.6

Data obtained with four chimeras, representative of 11 tested, are shown. 95 d after reconstitution with 2 × 10⁷ PBMC (donors are identified by capital letters in parentheses), the mice were killed and single-cell suspensions from lungs, liver, and spleen were pooled. The chimera-derived cells were plated out at 10⁶ total cells/well (0.3–2 × 10⁵ human T cells per well, as defined in retrospect by FACS®). Anti-CD3 antibody, rIL-2, and APC (T cell–depleted, irradiated, human PBMC, syngeneic to the donor) were added to the cultures as indicated (for further details see Materials and Methods). These primary in vitro cultures (day 0 results) were labeled with [³H]thymidine, harvested, and counted on day 5. Results (mean ± SD) are expressed as ³H incorporation (cpm × 10⁻³) of triplicate cultures. In parallel to the primary proliferation assay, human T cell lines were initiated from the individual chimeras (details for the initiation culture and the maintenance of the lines are described in Materials and Methods). The lines were retested after 15, 40, and 68 d of culture under the conditions indicated in a standard proliferation assay. Anergy in T cells was also seen when the chimeras were tested 41 and 176 d after injection of human cells.

human stem cell engraftment and differentiation. This notion is supported by the finding of others that after grafting SCID mice with fetal human liver and thymus, human hematopoiesis does occur within but does not occur outside the human transplant (4, 7).

The data in this report do not support the notion that the human leukocytes in the chimeras represent certain long-lived cell lineages which simply persist in the mice (possibility II). We found evidence for an impressive expansion of the human leukocytes in the murine host. In the first 20 d after intraperitoneal injection of 10×10^6 adult or cord blood cells, 0.01×10^6 human leukocytes (0.1%) were recovered from the local site and no human cells were detected in other organs of the recipients (Table 1). This initial disappearance of the injected cells could be due to lack of survival of the vast majority of human leukocytes and/or their dissemination in the mouse. Subsequently, the number of the human leukocytes gradually increased in all organs of the mice and usually peaked on day 50–70 after injection (Table 1). At this time point up to $50–100 \times 10^6$ human cells (5–10 times the inoculum) were recovered from lungs, liver, and spleen (Fig. 2 B) with an undefined number of human cells residing in those organs of the chimeras that cannot be readily analyzed by flow cytometry. The occurrence of high mitotic activity within the human leukocytes was confirmed by cell cycle studies (Fig. 5).

The phenotype conversion of the human T cells also argues against simple persistence of mature cells in SCID mice (possibility II): T lymphocytes recovered from cord blood–engrafted chimeras all expressed the phenotype CD45RA$^-$, CD45RO$^+$ (memory cells, Fig. 4) but all the T lymphocytes in the inoculum were CD45RA$^+$,CD45RO$^-$ (virgin cells, our own data, and reference 37). Similarly, after injection of adult PBL, consisting of roughly equal numbers of memory and virgin T cells (our own data and reference 37, 38), only CD45RA$^-$,CD45RO$^+$ T lymphocytes were detected in the mice (Fig. 3). Furthermore, MHC class II (HLA-DR)-positive T cells, that are virtually absent in cord blood and represent a minor population in adult blood, constituted the vast majority of T lymphocytes in the chimeras (Fig. 3 K, Fig. 4 J, K, and L).

Our data demonstrate that repopulation of SCID mice is dominated by two lymphocyte lineages. A heterogeneous population of human leukocytes was transferred into the mice, but only two human cell populations were recovered: α/β T cells and B lymphocytes. In all experiments, the non-T cell (CD3$^-$) population was identified as B cells (CD19$^+$ or CD20$^+$), and we did not detect human cells of a nonleukocyte origin in the chimeras (lack of CD45$^-$,H-2K^{d-} cells in Fig. 3 A).

One possible interpretation of our data is that SCID mice are repopulated with a subset of self-replenishing, mature, human lymphocytes, which proliferate in the secondary host independently of stimulation by antigen (possibility III). For example, it is conceivable that the total number of lymphocytes in the body is regulated (25). Thus, when lymphocyte counts drop beyond a critical level, clonal expansion within

the mature population would result until the normal cell number is reestablished. According to this model, a regulatory mechanism would induce human leukocytes to proliferate in SCID mice until they reach the regular density (300 million lymphocytes/mouse). This homeostatic model might not be compatible with our following data: (a) After reaching its peak value on days 50–70, the number of human lymphocytes steadily declines in the chimeras (Table 1). (b) As judged by cell cycle studies the human leukocytes still proliferate in the chimeras at time points, when the absolute number of human cells drops in the mice (data not shown). (c) The phenotype of the human T cells is rather uniform and does not change from early to late stages of chimerism. To resolve these data from the homeostatic model one would have to postulate that: the number of human leukocytes never reaches the critical level in SCID mice and therefore the proliferation-inducing stimulus persists; the potential for self-replenishing is exhaustible; the self-replenishing cell types have a short life span; and only CD45RA$^-$,CD45RO$^+$ T cells (along with some undefined B cells) are responding to this type of regulation. This T memory cell–like phenotype could either represent a distinct T cell subpopulation with self-replenishing capacity, or several T cell lineages might proliferate in response to the assumed homeostatic regulation and the memory cell–like phenotype would be expressed by cycling cells.

GVH reactivity (possibility IV) could also account for our data regarding the nature of the engrafted human T cells. There is clearly the potential for GVH reactions after transfer of mature human cells into SCID mice. In our hands SCID-splenic stimulator cells triggered in vitro proliferative responses by human PBL that were comparable in magnitude with classical, human MLR (M. Tary-Lehmann, unpublished data). Therefore, (at least under the in vitro conditions) SCID APC provide across the species barrier for all signals for activation of xenoreactive human T cells. Moreover, the vigorous human anti-SCID MLR suggests that the frequency of xenoreactive human T lymphocytes is in the range of alloreactive T cell populations (1–10% of the T cell pool) (39).

In addition the phenotype of the human T cells in the chimeras is consistent with lymphocytes that had been recently activated by antigen recognition (CD45RO$^+$, HLA-DR$^+$, Figs. 3 and 4). As with stimulated T cells, the lymphocytes in the chimeras do not express the lymph node homing receptor Leu8 (Fig. 3) (40–43). Accordingly, only few cells were found in the lymph nodes of the chimeras, but the majority of T cells were recovered in the lungs, liver, and spleen of the chimeras (Fig. 2 B). In fact, this tissue distribution reflects homing patterns of activated T cells. Such cells migrate preferentially to lungs, liver, and other peripheral tissues (19), but, lacking the Leu8 homing receptor, do not migrate to lymph nodes (42, 43). Under conditions of chronic T cell stimulation, e.g., during GVHD, T cells do not express increased levels of IL-2 receptor (44–46). Consistent with this observation, the human T cells in the chimera were negative for this activation marker (data not shown).

Given that the assumption was correct that xenoreactivity is the basis for selection and expansion of human T lympho-

cytes in the chimeras, why then are there no manifestations of GVHD? Based on current concepts about alloreactive T cell recognition, the following scenarios can be envisaged for the xenoreactive constellation in SCID human chimeras. First, xenoreactive human T cells recognize native murine MHC (H-2) molecules on SCID APC, probably involving presentation of peptides of either murine or human origin (47). To be stimulatory in this context, SCID APC had to express sufficient levels of H-2 antigens and accessory molecules (in the absence of endogenous lymphokines, expression of these molecules might be low in SCID mice). Furthermore, antigen presentation had to be functional across the species barrier, i.e., the murine APC had to be able to also provide the "second signal" which is known to be required for T cell activation beyond T cell receptor occupancy (48). It is unknown which of these conditions are met when SCID mice are engrafted with human PBL. For example, in the absence of the costimulatory signal, abortive T cell responses could result (48, 49). This type of T cell stimulation could lead to anergization of the xenoreactive human T cell population in the chimeras (see below).

The second possibility for the xenoreactive constellation in SCID human chimeras is that human T cells recognize murine antigens (H-2 or other gene products) in a processed form presented by the autologous APC, i.e., the xeno–response would be restricted to the donor's HLA molecules (50). It is important that the targets of this type of xenoreactivity would be human but not murine cells, and that the outcome would be self-destruction by donor cells rather than an attack against host tissues. This model could account for both the lack of GVHD in the chimeras, as well as the self-limiting nature of the repopulation of SCID mice with human lymphocytes reported here. The requirement of human APC for the anti-SCID proliferative response by the chimera-derived T cell lines (data not shown) might also support this latter model.

The human T cells, freshly isolated from the chimeras, did not respond to stimulation by anti-CD3 antibody or PHA as measured by proliferation (Table 3) and IL-2 secretion (data not shown). However, the T cells proliferated in the presence of IL-2. Thus we succeeded to establish long-term human T cell lines from hu-PBL-SCID mice. The lines retained the state of unresponsiveness for 15 to 40 d, after which they became responsive to triggering via the TCR (Table 3). This type of reversible anergy has been observed after Th cell stimulation, in the absence of the second signal (48, 49). It is

significant that antigen presentation by T (51, 52) and also by B cells (53, 54) has been demonstrated to induce anergy. Since T and B cells are the only human leukocytes we detected in the chimeras, and, since both cell types express MHC class II molecules in the mice (Figs. 3 and 4), HLA-restricted recognition of murine antigens in hu-PBL-SCID mice is prone to induce anergy in the xenoreactive human T cell population. But, as stated above, H-2-restricted xenoreactive responses, which involve direct recognition of murine APC, might also lead to anergy if the second signal is not delivered across the species barrier. In vivo, induction of anergy can be preceded by an initial phase of proliferation which is followed by contraction of the clonal size (55). Thus, the kinetics of repopulation of SCID mice with human leukocytes (Table 1) might be consistent with xenoreactive human T cell responses.

After the T cell lines overcome the anergic state, most lines (9 of 11) revealed proliferative responses to SCID splenic stimulator cells. The lines that did not respond consisted mainly of CD8$^+$ human T cells. These data clearly demonstrate that proliferative, xenoreactive human T cells were present in the majority of the chimeras. However, it remains unclear whether xenoreactive T cells constituted the dominant population in the mice. Also, the specificity of the CD8$^+$ cells has not been defined yet. The complete lack of proliferative response to anti-CD3 triggered activation in freshly isolated cells from the chimeras (Table 3) demonstrates that the vast majority of human T cells were refractory to stimulation. This might suggest that most of the T cells in the chimeras are anergized as a consequence of their xenoreactive antigen specificity. However, GVH reactivity is associated with a severe, generalized immunodeficiency which has been attributed to non–antigen-specific suppression mediated by CD8$^+$ and CD4$^+$ T cells (56, 57). In the latter case, a minor population of xenoreactive T cells could reversibly abolish the response of a major, nonxenoreactive T cell population. Therefore, in conclusion, though we have clearcut evidence for xenoreactivity in hu-PBL-SCID chimeras, we cannot formally define whether the human immune system in the mice is characterized by (a) a dominant, self-replenishing T cell population that proliferates in the absence of antigenic stimulation and has a minor xenoreactive component; or (b) whether xenoreactivity is the major, if not only stimulus for repopulation of SCID mice with human T cells. Currently we are performing experiments to distinguish between these possibilities.

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