Anti-SCID Mouse Reactivity Shapes the Human CD4+ T Cell Repertoire in hu-PBL-SCID Chimeras

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Summary

Injecting human peripheral blood mononuclear cells into severe combined immunodeficient (SCID) mice results in long-term engraftment of human lymphocytes, of which >98% are phenotypically mature, activated T cells. Here we have characterized the human T cells that populate such hu-PBL-SCID chimeras. We report that these human T cells do not mobilize Ca^{2+} after CD3 stimulation, i.e., their T cell receptor (TCR)–mediated signal transduction is deficient. Chimera-derived human T cells do not secrete lymphokines or undergo blastogenesis after CD3 stimulation, but proliferate in response to interleukin 2 (IL-2), defining the chimera derived human T cells as anergic. Anergy was seen in both the CD4+ and the CD8+ subpopulations. We established human T cell lines from chimeras. These T cells retained their anergic state for 1–2 mo in culture, after which they simultaneously regained the ability to mobilize Ca^{2+}, secrete lymphokines, and to undergo blastogenesis following stimulation via the TCR. Once regaining proliferative responsiveness to CD3 stimulation, these CD4+ T cell lines displayed anti-SCID mouse reactivity and showed no specificity for recall antigens. All CD3-responsive CD4+ T cell clones obtained from such lines were SCID mouse specific, recognizing native major histocompatibility complex class II products on the murine cells. In contrast, chimera-derived human CD8+ cell lines and clones did not display detectable anti–mouse reactivity. The data show that the human T cell system in long term hu-PBL-SCID chimeras is nonfunctional due to both anergy and the limitation of the CD4+ repertoire to xenoreactive clones. The data suggest that long-term hu-PBL-SCID chimerism represents an atypical graft-versus-host reaction in which the human effector T cells become anergic in the murine environment.

Considerable effort has been made to create animal models in which functions of the human immune system can be studied under experimental conditions in vivo. An important step in this direction was the demonstration that SCID mice can be stably grafted with human lymphoid cells (1–4). Long-term chimerism was achieved in SCID mice by injection with peripheral blood mononuclear cells (hu-PBL-SCID mice) (1) and by graftment with fetal lymphoid tissues (SCID-hu mice) (2, 3). In both models it is well established that human lymphocytes populate the mice in considerable numbers, but the biology of chimerism remains to be elucidated.

We have been characterizing the human immune system in hu-PBL-SCID mice. We (5) and others (6–11) have shown that >98% of the human cells that populate the chimeras are T cells that uniformly express a mature (CD4+ or CD8+) and activated/memory (CD45R0+, HLA-DR+)

phenotype. Cells freshly isolated from chimeras were found not to proliferate after stimulation with anti-CD3 antibody but to respond to IL-2. This finding provided indirect evidence that human T cells in the chimeras are nonfunctional and possibly anergic. Furthermore, we have shown that, when cultured in the presence of IL-2, the chimera-derived human T cells reacquired responsiveness to CD3 stimulation, at which time they also proliferated to SCID stimulator cells.

Thus, our previous findings raised two questions fundamental to the understanding of hu-PBL-SCID chimerism. First, is there direct evidence for the anergic state of human T cells in the chimeras? Second, does the anti-SCID reactivity of chimera-derived human T cell lines indicate selection of xenoreactive T cell specificities in the murine host?

Here we report on experiments that resolve both issues. First, we demonstrate that, in addition to their proliferative unresponsiveness, chimera derived human T cells can not be

induced to secrete lymphokines by CD3 stimulation and that the unresponsiveness is manifested at the level of signal transduction; chimera-derived human T cells do not mobilize Ca^{2+} after TCR cross-linking. These data provide direct evidence for an anergic state of human T cells in hu-PBL-SCID chimeras. Second, we demonstrate that all functional CD4$^+$ T cell clones isolated from independent chimera-derived T cell lines were anti-SCID mouse–specific, implying that xenoreactivity is the major stimulus shaping the human, CD4$^+$ T cell repertoire in hu-PBL-SCID chimeras.

Materials and Methods

Subjects. Donors were 12 healthy subjects between the ages 22–45 (11 males, 1 female). Two of these donors had not been infected with EBV as judged by a lack of serum antibodies for EBV.

Mice. CB.17 scid/scid (SCID) mice were bred at the University of California at Los Angeles (UCLA). CB.17, BALB/c, BALB/k, and B10.D2 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). BALB/b and B10.GD mice were a gift of Dr. Eli Sercarz (UCLA).

Grafting Mice with PBL and Recovery of Human Cells from hu-PBL-SCID Mice. We followed the protocol that we published previously (5). Briefly, 20 × 10^6 PBL were injected intraperitoneally and 30–90 d later spleen, liver, and lungs were collected. Single cell suspensions of these organs were obtained by gently teasing the tissue in sterile medium and passing the disrupted tissue through a sterile wire mesh. Cells from the peritoneal cavity were recovered by repeated injection and aspiration of 5 ml RPMI medium.

Flow Cytometric Analysis and Antibodies. Two-color flow cytometric analysis was performed as described previously (5). The following antibodies, specific for human cell surface markers, were purchased from Becton Dickinson & Co. (Mountain View, CA): CD3 (FITC and PE); CD4 (FITC and PE); CD8 (PE); CD19 (PE); CD20 (PE); CD45 (FITC); and TCR-α/β (FITC); and TCR-γ/δ (FITC). Mouse isotype controls and anti-I-A^d mAb (clone MK-D6) were also obtained from Becton Dickinson & Co. Biotinylated anti-H-2K^d mAb was purchased from PharMingen (San Diego, CA). PE-conjugated streptavidin was from Caltag Laboratories (San Francisco, CA). Murine mAbs, specific for human TCR variable regions, all FITC conjugated, were purchased from T Cell Sciences, Inc. (Cambridge, MA). These included mAbs specific for βV5(a), βV5(b), βV5(c), βV8(a), βV12(a), βV12(a), βV6(a), and αV2(a).

Ca^{2+} Flux Studies. Ca^{2+} flux analysis was performed as described in detail elsewhere (12). Biotinylated OKT3 at 1.25 μg/ml was added after loading the cells with 5 mM Indo-1 solution (Molecular Probes Inc., Eugene, OR), and then staining them with CD45 FITC. After ~2.5 min, 100 μl of 500 μg/ml egg white avidin (Sigma Chemical Co., St. Louis, MO) was added to the vial. The reaction was measured for an additional 3–5 min.

Proliferation Assays with T Cells Freshly Isolated from Chimeras. Cells were plated into 96-well flat-bottomed plates. The number of human cells/well was calculated in retrospect, after the percentage of human cells (usually 20–50%) had been established by FACS® analysis (Becton Dickinson & Co.). Subsequently, fresh autologous human APC were added, after being irradiated (3,000 rad) and T cell depleted by rosetting with sheep erythrocytes (T dep. PBL)[1] (13). Either such T dep. PBL or unseparated PBL were used for APC,

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[1] *Abbreviations used in this paper:* PPD, purified protein derivative; T dep. PBL, T cell depleted by rosetting with sheep erythrocytes.

as indicated. Anti-CD3 mAb (Orthoclone OKT3; Ortho Pharmaceutical Corp., Raritan, NJ) was used at 1 μg/ml. Recombinant human IL-2 (a gift of Sandoz Ltd., Basel, Switzerland) was used at 10 ng/ml or as specified in Fig. 1. The culture medium consisted of DMEM with 10% FCS, 1% glutamine, 1% nonessential amino acids, and 3 × 10^{-5} M 2-ME. After 5 d of culture, 1 μCi of [^{3}H]thymidine was added to each well. 18 h later the plates were harvested and the incorporation of the label was counted by liquid scintillation.

Generation of Human T Cell Lines from hu-PBL-SCID Mice. We have established cell lines following three protocols. In all of these protocols, we started with a pooled single cell suspension from spleen, lungs, and the liver of a single chimera at 4 × 10^6 cells/well in a 24-well plate suspended in 2 ml of medium containing 10 ng/ml of rIL-2. The medium was as described for the proliferation assay above. This is protocol 1. In protocol 2, T dep. PBL (10^6 cells/ml) and anti-CD3 mAb (1 μg/ml) were added at the initiation of the culture. In protocol 3, the human cells were positively sorted on a FACStar^{Plus}® (14) before initiation of the T cell lines. These purified, chimera-derived human cells were plated in 96-well plates at 4 × 10^5 cells/well along with T dep. PBL (10^5 cells/well). IL-2 and anti-CD3 mAb were added as in protocol 2. In all three protocols, cells were fed weekly with fresh DMEM medium containing rIL-2 (10 ng/ml). After 1 mo in culture, the cells were restimulated according to the original protocol and maintained in IL-2 for another month. This process was repeated monthly for long-term culture. The cells were tested every 1–2 wk for proliferative responsiveness to anti-CD3 mAb. Cell lines usually became responsive after 1–2 mo in culture.

Cloning of Long-Term Chimera–derived T Cell Lines. T cell lines were cloned by limiting dilution (0.3 cells/well) in the presence of T dep. PBL (2 × 10^4/well), anti-CD3 mAb (1 μg/ml), and rIL-2 (10 ng/ml) in 20 μl DMEM medium (see above) using Terasaki microtiter plates (Robbins Scientific, Mountain View, CA) (15).

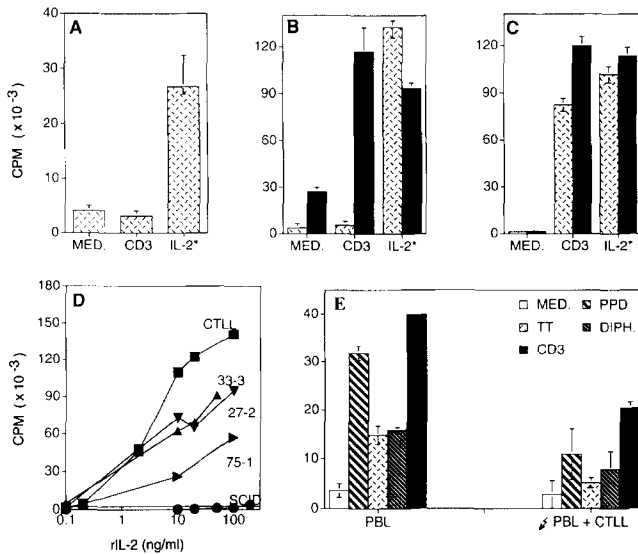*T Cell Proliferation Assay with Chimera-derived Human T Cell Lines or Clones.* Chimera-derived T cell lines or clones were plated at 3 × 10^4 cells/well in 96-well plates along with 10^5 T dep. PBL. Proliferative potential was assayed by stimulation with anti-CD3 mAb (1 μg/ml), tetanus toxin, diphtheria toxin (Wyeth Laboratories, Murietta, PA) or purified protein derivative of *Mycobacterium tuberculosis* (PPD; Evans Medical, Langhurst, UK) at 25 μg/ml. Anti–mouse reactivity was tested using irradiated (3,000 rad) spleen cells at 2 × 10^5 cells/well of the mouse strains specified. After 3 d of culture, 1 μCi [^{3}H]thymidine was added and incorporation of label was measured as described above.

Hemisplenectomy. Animals where anesthetized with pentobarbital (UCLA, Pharmacy) 40 mg/kg i.p., 10–15 min before surgery. Hemisplenectomy was performed as described in detail elsewhere (16).

Determination of Cytokine Production. Chimera-derived human T cells (3 × 10^4 cells/well) were stimulated with anti-CD3 mAb (1 μg/ml) in the presence of T dep. PBL (10^5 cells/well) in 24-well plates with 2 ml DMEM medium. Supernatants were collected 24, 48, and 72 h later and assayed for IL-2, GM-CSF, and IFN-γ by lymphokine-specific ELISA as previously described (17). The detection limit for IL-2 is 20 pg/ml, for IFN-γ is 150 pg/ml, and for GM-CSF is 50 pg/ml.

Cell-mediated Cytotoxicity Assay. 5 d before the actual ^{51}Cr release assay, effector cells (2 × 10^5 cells/well) were preactivated with anti-CD3 mAb (1 μg/ml) and T dep. PBL in 24-well plates, with 2 ml DMEM containing IL-2 at 10 ng/ml. On the day of the assay, the effector cells were washed and plated at 4 × 10^5–2.5 ×

Figure 1. Freshly isolated chimera-derived human T cells proliferate to IL-2 but do not proliferate to CD3 stimulation; long-term T cell lines regain responsiveness to TCR-mediated stimulation. (A) Proliferative responses by cells freshly isolated from chimeras. Single cell suspensions were prepared and pooled from the spleen, lungs, and liver of individual chimeras and the presence of human T cells (CD45+, CD3+) was tested by flow cytometry. The cell suspension shown here contained 26.8% human T cells and 73.2% murine (H-2K^{d}+) cells. (B and C) Proliferative response by chimera-derived T cell line after 24 (B) and 36 (C) d in culture. In B and C murine cells were undetectable (<0.1%). The chimera-derived cells were assayed in the presence of medium alone (MED), anti-CD3 mAb (1 µg/ml), or recombinant IL-2 (10 ng/ml). Cultures in (A) were supplemented with irradiated, T cell–depleted PBL as a source of autologous human accessory cells. In (B) and (C) irradiated, T cell–depleted PBL (open bars) or irradiated unseparated PBL (solid bars) were added as indicated. The data shown were obtained testing T cells from a single chimera. Bars represent SD of [3H]thymidine incorporation in triplicate cultures. These results are representative for 10 chimera derived cell lines that originated from four human donors. (D) Recombinant IL-2–induced proliferation was measured in cells derived from chimeras 33-3, 75-1, and 27-2 and in CTLL cells. The 33-3 cells were freshly isolated from chimera 33-3, contained 57% murine (H-2K^{d}+) cells and 43% human T cells (CD45+, CD3+). Similarly isolated cells from uninjected SCID mice (SCID) did not proliferate to doses of IL-2 to which 33-3 was highly responsive. Cells 75-1 were obtained by FACS® sorting for human CD4+ cells immediately after the isolation from the chimeras and were >99% CD3+. 33-3 and 75-1 did not proliferate to CD3 stimulation (not shown). Cell line 27-2, after being cultured for 161 d, contained no detectable murine cells and proliferated to CD3 stimulation. Cells of each type were present in the IL-2 assay at 2 × 10^5 cells/well. (E) Bystander proliferation of CTLL cells to IL-2 secreted by irradiated PBL. The data on the left of the panel show the results of a standard proliferation assay with unirradiated PBL of a human donor in response to: medium, purified protein derivate of M. tuberculosis (PPD), tetanus toxoid (TT), diphtheria toxin (DIPH), or anti-CD3 mAb. On the right of the panel, irradiated (3,000 rad) PBL of the same donor were tested under identical conditions, except that CTLL cells (2 × 10^4 cells/well) were added. 2 d later, the cultures were pulsed with [3H]thymidine for 12 h. Recall antigens or anti-CD3 mAb did not induce incorporation of label above background in irradiated PBL or CTLL cells when tested separately or when the irradiated PBL were cultured with CTLL in the absence of additional antigen (data not shown). The results in E were confirmed testing PBL of two additional human donors; also, in those experiments, both the pattern and magnitude of CTLL-bystander proliferation closely reproduced the response by the unirradiated PBL.

10^4 cells/well in U-bottomed, 96-well plates. Target cells consisted of Con A-activated (72 h, 3 µg/ml) spleen cells of CB17 mice that are congenic to SCID. Alternatively, as indicated, cell lines A20 and J774 (both H-2^d) were used. On the day of assay 10^7 target cells were labeled for 1 h with 200 µCi ^{51}Cr at 37°C, washed, and added at 10^4 cells/well to the effector cells. The Cr release assay was performed as described previously (18).

Results and Discussion

Chimera-derived Human T Cells Proliferate in Response to IL-2 But Not to Anti-CD3 Stimulation. Cells freshly isolated from chimeras consisted of 10–70% CD45+, CD3+ cells (human T cells), with the remaining cells being H-2K^d+ (cells of SCID mouse origin) (5). When such cell suspensions were challenged with anti-CD3 antibody, no proliferative response was induced (data not shown). Since such cell suspensions contained no detectable human macrophages and usually <<2% human B cells (5, 6, 8) the apparent unresponsiveness of the chimera-derived human T cells might have resulted from the absence of functional human APC. To test this possibility, we supplemented the cultures with autologous human PBL. Addition of irradiated, T cell–depleted APC did not render freshly isolated chimera-derived human T cells responsive to CD3 stimulation. However, the cells proliferated with IL-2 (Fig. 1 A).

To characterize these chimera-derived human T cells in depth, we established a total of 42 independent chimera-derived human T cell lines, from six different human donors. We systematically used three protocols for establishing the cell lines to control for the possibility of in vitro selection during tissue culture. First, chimera-derived cells were grown on IL-2 alone. Second, in addition to IL-2, syngeneic, irradiated, feeder cells and anti-CD3 mAb were added. Third, chimera-derived human cells were sorted to remove contaminating murine cells (see Materials and Methods) and then expanded with IL-2, anti-CD3 mAb, and syngeneic feeder cells. Importantly, all three protocols resulted in human T cell lines with identical properties, as described below.

When chimera-derived human T cell lines were tested for their responsiveness to CD3 stimulation, conflicting results were obtained depending upon whether T dep. or bulk PBL were used as a source of APC. All chimera-derived T cell lines were unresponsive to CD3 stimulation when tested with T cell–depleted APC but proliferated in the presence of unseparated PBL (Fig. 1 B). T cell lines that had been in culture for over 2 mo all responded to CD3 stimulation with both types of APC (Fig. 1 C).

Since the IL-2 response in chimera-derived cells (Fig. 1 A) might have resulted from proliferation by contaminating murine cells rather than by the human T cells, and because of the apparently conflicting, APC-dependent data with CD3 stimulation (Fig. 1 B), we performed further experiments to clearly establish the proliferative phenotype of the chimera-derived human T cells. Should the chimera-derived T cells be responsive to IL-2 but unresponsive to CD3 stimulation, this would suggest that these T cells were in an anergic state.

First, we determined whether the IL-2 response seen with

chimera-derived cells resulted from blastogenesis of the human or the murine cells. Cells derived from uninjected SCID mice did not proliferate in response to 10 ng IL-2/ml, the concentration used to test IL-2 reactivity in chimera-derived cells, and were barely stimulated at high concentrations of IL-2 (<100 ng/ml) (Fig. 1 D). In contrast, cells freshly isolated from chimeras (which contained human T cells and murine cells) as well as sorted human T cells from chimeras (which did not contain detectable murine cells), both of which were unresponsive to CD3 stimulation, proliferated vigorously to IL-2 (Fig. 1 D). Freshly isolated chimera-derived human T cells (33-3, 75-1), and long-term chimera-derived T cell lines showed comparable dose response characteristics and their sensitivity to IL-2 was comparable to the IL-2 sensitive indicator cell line, CTLL. These data clearly show that the IL-2 responsive population in chimera-derived cell suspensions are human T cells, rather than murine cells.

Next, we resolved the apparently conflicting data on the APC dependency of the CD3 responsiveness of chimera-derived T cells (Fig. 1 B). Since both, T dep. PBL and unseparated PBL functioned equally well as accessory cells for CD3 stimulation of long-term chimera-derived T cell lines (Fig. 1 C), it was unlikely that the lack of responsiveness of freshly isolated T cells in the presence of T cell–depleted APC (Fig. 1, A and B) resulted from deficient accessory cell function. We therefore tested the possibility that irradiation did not abrogate the ability of T cells in the unseparated PBL population to secrete IL-2. In this scenario the highly IL-2–sensitive, chimera-derived T cells (Fig. 1 D) would proliferate in a bystander fashion to the IL-2 that was released by the T cells in the irradiated PBL when the latter were stimulated by anti-CD3 mAb. We challenged irradiated PBL with anti-CD3 mAb and tested whether they promoted proliferation of the IL-2–sensitive indicator cell line CTLL (CTLL showed a comparable dose response characteristic to IL-2 as chimera

derived human T cells, Fig. 1 D). Vigorous bystander proliferation of CTLL cells was measured (Fig. 1 E). Beyond the polyclonal stimulation with the anti-CD3 mAb, even exposure of irradiated PBL to recall antigens induced sufficient IL-2 secretion to mediate CTLL-bystander proliferation. Strikingly, when irradiated PBL were challenged with the recall antigens tetanus toxoid, purified protein derivate of M. tuberculosis and diphtheria toxin, bystander proliferation of CTLL cells exactly reproduced the pattern of proliferation to these antigens by the unirradiated PBL (Fig. 1 E). Hence, the proliferative response by the highly IL-2–sensitive chimera-derived T cells to anti-CD3 stimulation in the presence of unseparated PBL (Fig. 1 B) represents IL-2–driven bystander proliferation by the chimera-derived human T cells rather than TCR triggered blastogenesis by these cells.

Collectively, the data in Fig. 1 establish that freshly isolated chimera-derived T cells and T cell lines cultured for <30 d are responsive to IL-2 but unresponsive to CD3 stimulation, i.e., they display a proliferative phenotype that is characteristic of anergic T cells (19). Additionally, these data resolve a controversy in the field, regarding the functionality of the chimera-derived human T cells, highlighting the importance of using T cell–depleted irradiated APC in proliferative assays to avoid false positive results.

Chimera-derived Human T Cells Are Deficient in Cytokine Production. We tested whether the chimera-derived human T cells also qualify as anergic cells on the basis of defective cytokine production (19). We did not detect IL-2, IFN-γ, or GM-CSF in supernatants of anti-CD3 stimulated chimera-derived T cells that were nonproliferative to this stimulus (Table 1). This deficiency was seen both with freshly isolated cells and with cell lines during the first month in culture. The cell lines that we tested contained considerable numbers (up to 80%) of CD8+ cells, in addition to CD4+ cells. Since "normal" CD8+ cells also secrete IFN-γ when stimulated

Table 1. *Freshly Isolated Chimera-derived Human T Cells and T Cell Lines That Are Nonproliferative to CD3 Stimulation Are Deficient in Cytokine Production*

	CD3 Stimulation*									
	Donor 1					Donor 2				
			CDHT cells‖					CDHT cells		
Cytokine	PBL	APC‡	Fresh	Non prol.§	Prol.§	PBL	APC	Fresh	Non prol.	Prol
IL-2	292	<20¶	<20	<20	59	799	<20	<20	<20	119
IFN-γ	3,404	<150¶	<150	<150	639	6,385	<150	<150	<150	3,366
GM-CSF	142	<50¶	<50	<50	84	569	<50	<50	<50	131

* Supernatants from cultures were collected 24 h after stimulation for IL-2 and 48 h for IFN-γ and GM-CSF measurements and results are expressed in pg/ml.
‡ Autologous irradiated T dep. PBL.
§ Nonproliferative or proliferative to CD3 stimulation.
‖ Chimera-derived human T cells and autologous irradiated T dep. PBL.
¶ Below detection limit of the ELISA. Positive results are underlined.

via the TCR (20), these data show that the deficiency in cytokine production applies to both CD4$^+$ and CD8$^+$ chimera-derived human T cells.

As chimera-derived T cell lines regained their proliferative responsiveness to CD3 stimulation, they also started to secrete IL-2, IFN-γ, and GM-CSF (Table 1). Production of IL-2, INF-γ, and GM-CSF was seen in purified CD4$^+$ and CD8$^+$ cell lines alike, both of which were initially unresponsive to CD3 stimulation (data not shown). Therefore, freshly isolated chimera-derived human CD4$^+$ and CD8$^+$ T cells exhibit a reversible deficiency in cytokine production, another feature of anergic T cells (19).

Chimera-derived Human T Cells Are Deficient in Activation-dependent Ca^{2+} Mobilization. We tested whether Ca^{2+} mobilization, one of the earliest events in TCR-induced T cell activation, was operational in these cells. Anti-CD3 mAb treatment failed to induce Ca^{2+} flux in freshly isolated chimera-derived cells and chimera-derived T cell lines that were nonproliferative to this stimulus (Fig. 2 C). Since the samples tested contained both human CD4$^+$ and CD8$^+$ cells (Fig. 2), the inability to mobilize Ca^{2+} applies to both cell types. The chimera-derived human T cells exhibited Ca^{2+} flux when membrane signaling was bypassed by ionomycin treatment (Fig. 2 D). During long-term culture, the chimera-derived cell lines simultaneously regained Ca^{2+} mobilization and proliferative responsiveness to anti-CD3 stimulation (Fig. 2 E).

As we have previously shown, the level of CD3 and TCR-α/β expression are not decreased in chimera-derived human T cells (5). Therefore, in spite of binding to the TCR, the anti-CD3 mAb failed to mobilize Ca^{2+}, which demonstrates that the anergic state of chimera-derived human T cells results

from defective signaling via the TCR. Stimulation of chimera-derived T cells with Ca^{2+} ionophores and PMA, agents that bypass the TCR-mediated signaling pathway, did not induce lymphokine production (not shown). This suggests that, in addition to a block in TCR-mediated early activation events, such as Ca^{2+} mobilization, there is a more distal block as well. The deficiency in Ca^{2+} mobilization may be a generalizable feature of anergic T cells, since it has also been observed in unrelated models of anergy (21, 22).

The Human T Cells That Repopulate Chimeras Do Not Show Preferential TCR-V Gene Use. While engrafting SCID mice with human PBL, we noted an initial rapid decline in human cells followed by their considerable expansion in the adoptive host (Fig. 3). This dynamic is consistent with selection of lymphocyte subpopulations in the new environment. Dependent on the nature of the selection process, the "successful" T cell population may include a wide variety of clones, or only a few clones may establish themselves, similar to the oligoclonal repopulation of hu-PBL-SCID mice with human B cells (23). To study the T cell repertoire in hu-PBL-SCID chimeras, we defined the frequency of TCR-V gene positive T cells in the PBL population at grafting and we compared it with that in long-term chimeras. Using a panel of TCR-V gene-specific antibodies we found that the overall representation of T cells expressing a particular V gene was comparable in both populations (Table 2). Additionally, we hemisplenectomized chimeras to compare the frequency of TCR V gene positive T cells in a single chimera at two different time points. Although some shifts in the representation of V gene$^+$ populations occurred with time in individual mice, the polyclonal nature of their T cell repertoire was retained (Table 2). These data show that (a) the human T cells that
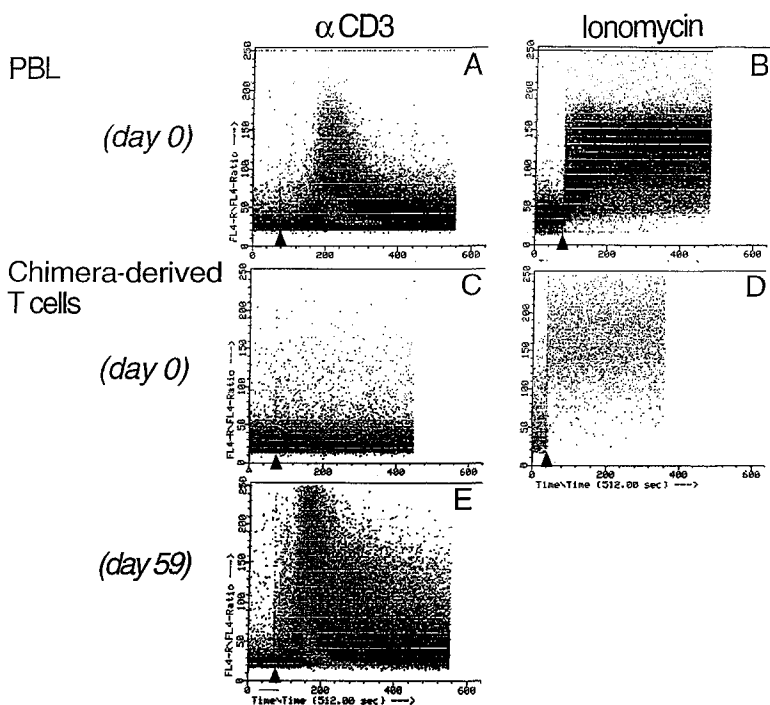
Figure 2. CD3 stimulation, as opposed to ionomycin treatment, does not induce Ca^{2+} flux in freshly isolated chimera-derived human T cells. (*A*) Subject's fresh PBL, (*C*) T cells freshly isolated from a chimera, and (*E*) a T cell line derived from the same chimera after 59 d in culture were challenged with anti-CD3 mAb (1 μg/ml) or ionomycin (2 mM), as indicated. The time point of stimulation is marked (▲). The results are representative for six chimera-derived T cell lines that originated from three different donors. Normal Ca^{2+} flux by PBL to CD3 or ionomycin stimulation are shown in panels *A* and *B*. Human T cells fresh from the chimera failed to show Ca^{2+} flux to CD3 stimulation (*C*) while these cells showed Ca^{2+} flux to ionomycin (*D*). On day 59, the chimera-derived T cell line first showed Ca^{2+} flux (*E*) and, also, a proliferative response to CD3 stimulation (data not shown). In the cell line younger than 52 d, no proliferative response was induced and results identical to *C* and *D* were obtained (data not shown). Ca^{2+} flux was measured in CD45 stained and gated cells (see Materials and Methods). Thereby, Ca^{2+} flux was measured only in human cells which is pertinent to *C* and *D* where a mixed population of murine and human T cells were tested (57% H-2K^{d+}, 43% CD45$^+$CD3$^+$; of the human T cells 22% were CD4$^+$ and 78% were CD8$^+$).
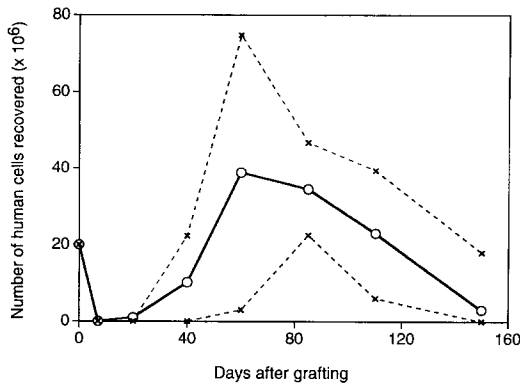
Figure 3. Kinetics of SCID mouse population by human T cells. Mice were injected with 2×10^7 PBL i.p. and, at the indicated time points after grafting, mice were killed and the number of human cells (CD45$^+$) was assessed by flow cytometry analysis in cell suspensions obtained from the peritoneal cavity, lungs, liver, thymus, and the spleen. The cumulative cell numbers recovered from these organs of individual mice were used to establish the mean (*solid line*) and 1 SD (*dotted lines*) for each time point. Data obtained from 50 chimeras that have been grafted with either of 10 human donors are shown: each data point was calculated from 3–11 chimeras. The human cells were <98% T cells (5). During approximately the first 20 d, human cells were detected in the peritoneal cavity only, where they persisted in low numbers (<2 × 10^6 cells). Around day 30, human cells appeared in lungs, liver, and spleen (5) where the vast majority of the human cells resided; human T cells occurred in lower numbers also in other organs of the chimeras (5), which we did not collect here. Therefore, the data accurately reflect the kinetics of repopulation but underrepresent the total number of human cells in the chimeras.

populate the mice express a wide array of T cell receptor genes, i.e., they are not mono- or oligoclonal; and (b) there is no strong selection for or against the TCR V genes tested.

Chimera-derived T Cell Lines Show No Reactivity for Recall Antigens. We also assessed the human T cell repertoire in hu-PBL-SCID mice using functional criteria, asking whether clonally expanded T cell specificities in the donor inoculum are also present in high numbers in the chimeras. For example, if the donor was immunized against tetanus toxoid and his PBL were reactive to it, would T cells specific for this recall antigen also be detectable in chimeras grafted with this donor's PBL?

Direct, functional assessment of the human T cell repertoire in the chimeras was impossible because of the anergic state by the chimera-derived T cells. We therefore studied the antigen responsiveness of chimera-derived T cell lines that had recovered from the anergic state after 1–2 mo of in vitro culture. When tested with T-depleted APC, none of these T cell lines responded to the recall antigens to which the donor's PBL responded (Table 3). Instead, we noted that anti-SCID mouse reactivity, which in PBL was comparable in magnitude to recall antigen responses (but much weaker than an alloresponse), prevailed in all chimera-derived T cell lines that contained >3% CD4$^+$ cells (39 of 42 T cell lines). Only three T cell lines, which consisted of >97% CD8$^+$ cells, failed to proliferate in the presence of SCID stimulator cells, although these cell lines proliferated to CD3 stimulation (Table 3).

Table 2. *Expression of TCR-V Genes by Chimera-derived Human T Cells Is Unbiased*

	CD3$^+$ cells expressing V gene								
	Donor A		Donor B		Donor C				
						Chimera M1 and M2			
TCR-V genes	PBL	Chimera	PBL	Chimera	PBL	day 83		day 119	
	day 0	day 56	day 0	day 106	day 0	M1	M2	M1	M2
					%				
βV5(a)	1.7	4.6	2.5	0.3	2.4	2.6	2.2	0.7	2.5
βV5(b)	0.8	0.9	1.0	0.3	0.6	3.6	1.9	0.0	2.6
βV5(c)	4.6	6.0	4.0	0.6	3.7	1.5	2.2	2.0	0.5
βV6(a)	2.0	1.4	2.8	1.1	2.5	2.7	1.9	1.1	1.6
βV8(a)	2.9	3.3	4.2	0.2	3.8	6.8	6.4	1.9	1.8
βV12(a)	1.6	3.4	1.4	1.2	0.8	7.3	5.0	2.8	2.6
αV2(a)	3.0	2.0	2.3	1.1	2.8	5.7	6.6	0.5	0.6

PBL of three human donors were stained with anti-CD3 mAb and with the listed mAbs specific for TCR-V gene products. Two-color flow cytometric analysis was performed to define the frequency of CD3$^+$ cells that express a particular V gene. On the same day (day 0) SCID mice were grafted with the PBL of these donors. At the indicated time points, the frequency of CD3$^+$, TCR-V gene$^+$ cells was redefined in the spleens of chimeras. From two chimeras M1 and M2, (grafted by donor C) one half of their spleen was surgically removed on day 83. On day 119 these chimeras were killed and the other half of the spleen was recovered. Flow cytometry was performed at both time points.

Table 3. *Unlike the Donors' PBL, Long-term CD4⁺ Chimera-derived Human T Cell Lines Do Not Respond to Recall Antigens But Proliferate to SCID Stimulator Cells*

Proliferation to (cpm):	Donor A		Donor B		Donor C	
	PBL	CDHT* (64% CD4⁺)	PBL	CDHT* (84% CD4⁺)	PBL	CDHT* (99% CD8⁺)
Medium	5,355	10,871	2,099	1,771	3,959	1,406
TT	14,799	13,054	11,587	1,443	13,054	3,344
PPD	31,796	8,146	31,075	2,440	6,234	666
SCID	13,160	63,224	22,623	218,545	6,875	3,702
αCD3	97,819	75,872	O.G.‡	174,198	125,323	170,441

Underlined SI >3.
* Long-term chimera-derived human T cell lines, tested with autologous irradiated T dep. PBL.
‡ Overgrown.

The three CD8⁺ chimera-derived T cell lines did not exhibit cognate lytic activity against H-2ᵈ target cells, but they were cytolytic when their TCR was linked to the target cells with PHA (Fig. 4). Therefore, the lytic machinery was functional in these CD8⁺ cell lines, but, apparently, they were not specific for murine antigens expressed by the target cells.

While these data are not sufficient to provide conclusions about the CD8⁺ T cell repertoire in chimeras, they do demonstrate considerable positive and negative selection at the CD4⁺ cell level. Since proliferative responses to antigens provide a measure of the frequency of antigen-specific CD4⁺ T cells, our data show that during chimerism, and/or in vitro culture, the frequency of CD4⁺ T cells specific for murine antigens increases considerably whereas the frequency of recall antigen-specific CD4⁺ cells decreases beyond the limit of detection (cloning of these T cell lines confirmed this notion, see below).

Characterization of CD4⁺ Chimera-derived T Cell Clones.
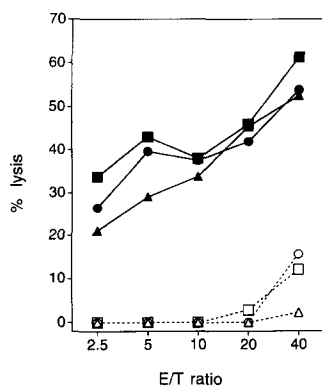
Figure 4. Chimera-derived CD8⁺ human T cell lines are noncytolytic to CB.17 Con A blasts, but kill these blasts in the presence of PHA. Three chimera-derived T cell lines (■, ●, ▲) that were CD8⁺ and regained proliferative responsiveness to CD3 stimulation were tested in a standard 4-h ⁵¹Cr release assay (see Materials and Methods). Cytotoxicity was tested against CB.17 Con A blasts (*H-2ᵈ*, congenic to SCID) in the absence (*open symbols*) or presence of 3 μg/ml PHA (*solid symbols*). Similar results were obtained using *H-2ᵈ*-expressing tumor cells J774 and A20 as target cells and chimera-derived CD8⁺ clones as effector cells (not shown). The data are representative for two experiments performed.

We cloned chimera-derived T cell lines to establish the frequency of anti-SCID mouse–specific clones among them. If xenoreactivity was the driving force for engraftment of CD4⁺ human T cells, then all (or most) clones isolated from chimera-derived T cell lines should display anti-SCID specificity. Alternatively, xenoantigen-driven repopulation would be unlikely if these cell lines contained only a minor fraction of xenoreactive T cells (which could still be sufficient to account for the anti-SCID reactivity of the lines).

We cloned two cell lines in which the possibility of in vitro priming by murine cells was minimalized; one line was established with added anti-CD3 mAb, the other cell line had murine cells removed by cell sorting before initiation of the culture. The cell lines originated from different human donors. Of 58 and 45 CD4⁺ T cell clones obtained, respectively, 46 and 29 clones were responsive to CD3 stimulation. All these clones displayed anti-SCID mouse reactivity (Table 4). The remaining clones were refractory to stimulation with anti-CD3 mAb or with SCID spleen stimulator cells, possibly due to persisting anergy or to chromosomal aberrations due to the chronic stimulation in the secondary host. Hence, every functional CD4⁺ T cell clone we isolated displayed anti-SCID mouse reactivity.

With respect to fine specificity, these CD4⁺ clones fell into two major categories. The majority of clones were monospecific for H-2ᵈ (expressed by both SCID and BALB/c stimulator cells) and did not respond to congenic stimulator cells that differed in the H-2 haplotype (BALB/k:H-2ᵏ or BALB/b:H-2ᵇ). Of the 29 and 46 functional T cell clones obtained from the two donors, respectively, 22 and 36 exhibited this type of reactivity (sum of anti-I-Aᵈ- or anti-I-Eᵈ-specific clones in Table 4). These clones recognized polymorphic H-2ᵈ gene products, being specific for either the I-Aᵈ or I-Eᵈ molecule. Specificity for I-Eᵈ was defined by responsiveness to B10.D2 stimulator cells (I-Aᵈ, I-Eᵈ) and failure to respond to congenic B10.GD stimulator cells (I-Aᵈ, I-E(ᵇ)) that do not express an I-E molecule (Table 4). Specificity for I-Aᵈ was defined by responsiveness to B10.GD

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stimulator cells that was blocked by anti-I-A^d-specific mAb (Table 4).

17 of the CD4+ clones, that displayed reactivity to H-2^d, also displayed reactivity to BALB/k (H-2^k) and/or BALB/b (H-2^b) stimulator cells (Table 4). As before, this response was either I-A- or I-E-restricted, as defined by antibody blocking experiments. Unlike the first set of clones that were monospecific for H-2^d products, the latter clones also crossreactively recognized other allelic MHC products, in addition to H-2^d.

The finding that the xenoresponse of these clones was blocked by antibodies specific for murine MHC products and, furthermore, that this response occurred in the absence of added human APC, suggests that the clones directly recognized native H-2 antigens on murine stimulator cells. However, the specificity of T cell clones obtained from T cell lines may not fully characterize the xenoreactivity by CD4+ cells in the chimeras. Thus, in several of our chimera-derived T cell lines the anti-SCID proliferative response was significantly increased in the presence of autologous, human, T cell–depleted APC (not shown). This finding may simply result from costimulation by human accessory molecules or cytokines, but it may also reflect the occurrence of HLA restricted recognition of processed murine antigens in hu-PBL-SCID chimeras.

Characterization of CD8+ Chimera-derived T Cell Clones. We obtained nine CD8+ T cell clones. These clones proliferated to anti-CD3 mAb in the presence of autologous APC but did not undergo blastogenesis when challenged with SCID stimulator cells (not shown). We tested the CD8+ clones in a standard ^{51}Cr release assays for cytotoxicity to the following target cells: (a) CB.17 Con A blasts; (b) A20 cells;

Table 4. *Chimera-derived CD4+ Human T Cell Clones Are Specific for Murine MHC Class II Products I-A or I-E*

Name of clone	A9A12	A6C10	641C2
Clone's proliferation to (cpm)			
Medium	773	2,542	254
SCID (H-2^d)	11,709	699,692	238,425
BALB/c (H-2^d)	44,473	372,280	85,604
BALB/k(H-2^k)	650	2,585	61,609
BALB/b(H-2^b)	768	ND	ND
B10.GD(I-A^d, I-E^b)	21,555*	1,545	83,624*
B10.D2(I-A^d, I-E^d)	ND	197,835	83,142*
Specificity	αI-A^d*	αI-E^d	αI-A^d, k*
No. of clones of this type			
Donor 1:	21	1	7
Donor 2:	27	9	10

SI >3, underlined.
* Inhibited by anti-I-A^d mAb (MK-D6).

and (c) J774 cells. Although all these target cells shared the H-2^d haplotype with SCID mice and were sensitive to CD8+-mediated cytotoxicity, we observed no or only marginal lytic activity. However, all clones were cytolytic in the presence of PHA that mimicks antigen recognition (data not shown, results are identical to those obtained testing the CD8+ T cell lines in Fig. 4). Thus the CD8+ clones, like the three independent CD8+ chimera-derived T cell lines, were functional but provided no direct evidence for anti-H-2^d-specificity.

In Vivo vs. In Vitro Selection of Xenoreactive T Cells. Two lines of evidence provided in this report show that the human lymphocytes present in long-term hu-PBL-SCID chimeras do not constitute a functional human immune system in the recipient mice. First, all the human T cells, CD4+ and CD8+ T cells alike, are in a state of anergy upon their isolation from the chimeras. Thus, their proliferative phenotype (Fig. 1), their deficiency in lymphokine production (Table 1), and Ca^2+ mobilization (Fig. 2) are all features characteristic of anergic T cells (19). Second, our data strongly suggest that the human CD4+ T cell repertoire in chimeras is being selected for and confined to anti-SCID mouse reactive (xenoreactive) specificities (Table 3 and 4).

Our conclusion that xenoreactivity is the stimulus for shaping the CD4+ repertoire is based on indirect evidence, i.e., studies involving chimera-derived T cell lines and clones that have recovered from their state of anergy during long-term tissue culture in the presence of IL-2 (24, 25). However, six independent lines of inferential evidence all point to the same conclusion, that the selection of xenoreactive T cell specificities occurred in vivo.

First, selection of SCID mouse–specific T cells in vitro, owing to the presence of murine cells in the culture, would require a TCR-dependent process. However, signaling through the TCR is uncoupled in chimera-derived human T cells during the first month in culture (Fig. 2), by which time murine cell contaminants have been lost from the cell lines. The frequency of H-2K^d+ murine cells drops beyond the detection limit by FACS® analysis within the first 14 d of culture (Tary-Lehmann, M., unpublished observation).

Second, the anergic, chimera-derived T cell lines, which we tested repeatedly for responsiveness to CD3 stimulation and SCID mouse reactivity, simultaneously acquired a proliferative responsiveness to both stimuli. Importantly, this earliest response is comparable in magnitude to both stimuli, suggesting that the frequency of SCID mouse reactive T cells in these cell lines closely matches the frequency of anti-CD3–reactive CD4+ cells, even at the time point when the T cells have just regained reactivity (Table 3). This finding argues against a gradual selection process due to undetectably rare contaminating murine cells after the human T cells have recovered from their anergic state.

Third, to "mimic" a TCR-related selection process, we added anti-CD3 mAb to freshly isolated cells, and obtained SCID mouse–reactive CD4+ lines as well. The same reactivity was displayed by T cell lines that have been established after removal of murine cells by FACS® sorting. In fact, within

these "control" cell lines, every functional CD4$^+$ clone that we have isolated was anti-SCID mouse reactive (Table 4).

Fourth, the fact that, in the chimeras, the human T cells uniformly express an activated/memory phenotype (CD45 RO$^+$, HLA-DR$^+$) (5, 6, 9–11) also favors the notion of a (xeno-) antigen-driven process. Activated/memory cells are short lived in the absence of their cognate antigen (26). Thus, it is likely that only those memory cell specificities establish themselves in the mice that are stimulated by (xeno-) antigen recognition (Table 3). If repopulation was independent of antigen recognition, one would expect that long-lived T cell populations would persist in the mice, as they are thought to be naive/resting cells (27). For reasons that are unclear, phenotypically naive (CD45RA$^+$, HLA-DR$^-$) T cells do not persist in SCID mice. To the contrary, injection of cord blood that contains only T cells of the naive phenotype, results in phenotype conversion and repopulation by T cells that express activation/memory markers (5). This, too, strongly argues for a (xeno-) antigen-driven process.

Fifth, the anergic state of the human T cells in the chimeras is indicative of a (xeno-) antigen-driven engraftment. All previously defined mechanisms that lead to T cell anergy result from antigen recognition (19, 28–31). Since the transition of the human T cells from a functional to an anergic state occurs during chimerism (Fig. 2), it has to be mediated by antigens that the human T cells encounter in the mouse. The specificity of the chimera-derived T cell clones suggests that, in the case of CD4$^+$ cells, native murine MHC products are being recognized directly on the murine cells (Table 4). Apparently, this xenoantigen recognition on murine cells results in induction of anergy in the human T cells.

Finally, we can not envision a scenario in which human T cells that are highly responsive to SCID MHC-products in vitro (Tables 3 and 4) would persist, unaffected, in the chimeras for weeks. We conclude that the xenoantigen specificity of the long-term, chimera-derived CD4$^+$ T cell lines and clones fully characterizes the CD4$^+$ repertoire in hu-PBL-SCID mice. Our data suggest that graft-vs.-host reactivity is the driving force for the population of SCID mice by human CD4$^+$ cells.

Our data on the human CD8$^+$ cell compartment in the chimeras are more complex. On one hand, long-term chimera-derived human CD8$^+$ T cell lines and clones did not display detectable anti-H-2^d reactivity (Fig. 4). On the other hand, the human CD8$^+$ cells uniformly displayed an activated phenotype in the mice (5) and these lymphocytes also developed a long lasting anergy during chimerism, being deficient in Ca^{2+} mobilization (Fig. 2), lymphokine production (Table 1), and blastogenesis (Fig. 1). That the CD8$^+$ cells have been rendered anergic in the chimera is highly suggestive for antigen recognition (22) implying a (xeno-) antigen-driven process. In spite of the absence of a detectable reactivity to SCID spleen cells (Table 3) and H-2^d-expressing cell lines (Fig. 4), the chimera-derived CD8$^+$ cells could be specific for murine antigens that are expressed in a tissue-specific manner and presented in association with murine class I molecules in organs other than the spleen. It is also pos-

sible that, in the chimeras, human CD8$^+$ cells are selected that have low affinity for murine antigens: a low affinity TCR signal is known to partially activate T cells rendering them unresponsive to subsequent, optimal stimulation (28). Therefore, the activated, anergic state of these cells in the chimeras may suggest a xenoantigen-driven repopulation by human CD8$^+$ cells.

Conclusions on hu-PBL-SCID Chimeras. The anergy that we observe in the xenoantigen reactive, chimera-derived human T cells may be a consequence of deficient costimulation in the murine environment. Reminiscent of the T cell response to superantigens (32–34), in which costimulation is thought to be deficient, an initial extensive clonal expansion is triggered, followed by contraction of clonal sizes and anergy. However, initial expansion followed by contraction of clonal sizes and anergy has also been reported for mature, HY antigen–specific murine CD8$^+$ cells that were injected into HY expressing hosts (35). Therefore, anergy may be the outcome of a "frustrated" T cell response where the antigen can not be cleared and continued T cell stimulation results, as it is the case for anti–mouse-specific human T cells in hu-PBL-SCID chimeras. We have found that the human T cells are already anergic 4–6 wk after grafting (5), at which time the T cell pool expands in chimeras (Fig. 3) and at which time strong mitotic activity is seen by cell cycle analysis (5, 6). Therefore, the antigen recognition event that induces the anergic state in the T cell appears to be followed by extensive proliferation, i.e., induction of anergy precedes proliferation. At the peak of chimerism, $\sim$1 mo after grafting, the numbers of T cells recovered from organs of the chimeras can exceed the number injected by 10-fold (Fig. 3). The subsequent decline in numbers of human T cells in the chimera (Fig. 3) may correspond to the contraction of clonal sizes that is seen with the superantigen (32–34) and HY antigen (35) response.

The early transition of the xenoreactive human T cells from a functional to an anergic state, before the extensive proliferation by the lymphocytes, explains why overt symptoms of GVHD fail to develop in chimeras whose organs can contain up to 80% (xenoreactive) human T cells (5). However, it should be noted that mild symptoms of GVHD, possibly mediated by the first generation of still functional T cells, are not uncommon in chimeras. These include splenomegaly (9), followed by atrophy of the spleen (6), and spontaneous autoantibody production to red blood cells (36), all characteristic features of GVHD.

In summary, our data imply that hu-PBL-SCID chimerism represents a novel type of graft-vs.-host reactivity that is atypical due to the early development of anergy in the effector cells. Apparently, the hu-PBL-SCID model does not fulfill the promise to provide a functional human immune system in an experimental animal. However, this model contributes a powerful model for experimentation on human T lymphocytes in vivo if an immunocompetent T cell system is not a requirement, e.g., for the infection of human T cells with HIV (37). Also, it provides a unique model for studies of anergy in vivo. Future attempts to improve the model towards constructing a functional human immune system in

the mouse will have to overcome at least three of its deficiencies: the lack of human accessory cells in lymphoid organs of the chimeras to serve as APC; the induction of an antihost response; and the decay of non-xenoreactive T cells.

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