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IL-7 Enhances the Responsiveness of Human T Cells That Develop in the Bone Marrow of Athymic Mice

Eleanor C. Tsark, Mo A. Dao, Xiuli Wang, Kenneth Weinberg, and Jan A. Nolta

The beige/nude/xid/human (bnx/hu) model of human hematopoiesis provides a unique opportunity to study extrathymic human T lymphocyte development in an in vivo system. Purified human hematopoietic stem cells develop into mature T lymphocytes and immature progenitors in the bone marrow of athymic bnx mice. The human T cells are all TCRαβ+ and display a restricted TCRVβ repertoire. In the current studies, we examined the effects of systemic human IL-7 (huIL-7) administration on the phenotype and the activation status of the bnx/hu T cells. In the majority of the mice that did not have huIL-7 administration, a higher frequency of human CD3+CD8+ than CD3+CD4+ T cells developed in the bone marrow. This phenomenon is also frequently observed in human bone marrow transplant recipients. Extremely low levels of IL-2 were expressed by human CD3+ cells isolated from these mice, in response to PMA plus ionomycin and to CD3 and CD28 cross-linking. IL-4 was not expressed by cells exposed to either stimulus, demonstrating a profound inability of the bnx/hu T cells to produce this cytokine. Systemic production of huIL-7 from engineered stromal cells transplanted into the mice increased the human CD4 to CD8 ratios, and increased the ratio of memory to naive CD4+ and CD8+ T cells. The human CD3+ cells recovered from mice that had systemic huIL-7 and equivalent numbers of CD3+CD4+ and CD3+CD8+ cells in the marrow were still unable to produce IL-4 in response to any condition tested, but were capable of normal levels of IL-2 production following stimulation. The Journal of Immunology, 2001, 166: 170–181.

Understanding the process of extrathymic differentiation of T cells could be important to obtain immune reconstitution in adult bone marrow transplant recipients with low thymic function, without generation of autoreactive clones. The mechanisms regulating development and activation of extrathymically derived human T cells are difficult to study. Following bone marrow transplantation, the low level of thymic function in adults and peripheral expansion of mature T cells from donor or recipient can mask extrathymic development (1–4). The beige/nude/xid/human (bnx/hu) immune-deficient mouse xenotransplantation model provides a unique opportunity to study extrathymic human T cell development. In this system, bnx mice are used as recipients of purified human hematopoietic stem and progenitor cells. Long-term hematopoiesis (up to 18 mo post-transplantation) of the human stem cell inoculum is sustained by cotransplantation of human bone marrow stromal cells engineered to produce human IL-3 (huIL-3) (5–7). Since bnx mice are athymic, the differentiation of mature human T cells in the bnx/hu model occurs through extrathymic differentiation, potentially by mechanisms described by Strober and colleagues (8–10) in the bone marrow of athymic and euthymic murine transplant recipients. Stem cells purified from human bone marrow, devoid of mature T cells, develop into all myeloid lineages, erythroid and myeloid progenitors, B cells, and mature T cells in the bone marrow of bnx mice (5–7, 11–19). The phenotype of the human T cells is predominantly CD3+/CD4−/CD8+, with lower levels of CD3+/CD4+/CD8− cells. Occasionally populations of “double-positive” CD3+/CD4+/CD8− cells can be recovered from the bnx bone marrow. All of the T cells express CD3 and do not express the NK cell markers CD56 and CD57. The CD3+ cells all express TCRαβ and no substantial levels of TCRγδ cells have been detected. The TCRVβ repertoire is highly skewed, and is not representative of the donor’s PBL distribution, suggesting interaction with the murine microenvironment (5).

To generate a functional model of the human immune system in a murine xenograft model, the human T cells must have the capacity to be activated in the mice when an appropriate Ag is presented. Optimal stimulation of human T cells requires two signals. The TCR must bind to an MHC molecule (either class I for CD8+ cells, or class II for CD4+ cells) bearing an associated peptide in the binding cleft. In addition, a costimulatory signal must be delivered to prevent a state of anergy or hyporesponsiveness from occurring. A well-characterized costimulatory signal is the binding of CD80 (B7-1) or CD86 (B7-2) on an activated APC to the T cells, or class II for CD4+ cells) bearing an associated peptide in the binding cleft. In addition, a costimulatory signal must be delivered to prevent a state of anergy or hyporesponsiveness from occurring. A well-characterized costimulatory signal is the binding of CD80 (B7-1) or CD86 (B7-2) on an activated APC to the TCR on the T cell (reviewed in Refs. 20 and 21). The combined signal results in IL-2 production and T cell proliferation. In the current studies, we mimicked engagement of the TCR and CD28 using the method developed by June and colleagues (22), stimulation of the cells by beads linked to anti-CD3 and anti-CD28 Abs. This system provides a relatively physiological stimulation, which can be compared with the nonphysiological strong stimulus provided by treatment of the T cells with PMA and ionomycin.

The current studies define the activation status of human T cells generated from purified human hematopoietic stem/progenitor
cells through extrathymic mechanisms in the bone marrow of bnx mice. The extrathymically derived T cells were found to be hyporesponsive and to have a limited capacity to produce IL-2, but not IL-4, following stimulation. The systemic production of human IL-7 (huIL-7) in the mice, in addition to the huIL-3 that is required to sustain the xenograft, significantly increased levels of human CD4+ cells. In mice that had systemic huIL-3 and equivalent levels of human CD4+ and CD8+ cells, IL-2 production in response to a physiological stimulus was restored. At this point, it is unknown whether the unique activation state that we have observed in the extrathymically derived T cells is unique to the bnx/hu model, or is a more general property that represents development in the bone marrow compartment.

Materials and Methods

Immune-deficient mice and cotransplantation

Six- to 8-wk-old homozygous bnx mice, bred at Childrens Hospital of Los Angeles (Los Angeles, CA), were used for all studies. Cotransplantation of human hematopoietic progenitor cells (500,000 CD34+ cells, isolated using immunomagnetic selection by Dynabeads (Dynal, Oslo, Norway) or 2,000 CD34+CD38- cells (purified by FACS acquisition)) and human bone marrow stromal cells engineered to secrete huIL-3 or a combination of huIL-3 and huIL-7, was performed as previously described (5–7, 11–19). A total of 1,000,000 marrow stromal cells were cotransplanted with the stem cells in each experiment. Mice were killed by 75% CO2/25% O2 narcosis 8–12 mo after transplantation. Bone marrow was flushed from the four long bones of the hind legs, and used immediately for FACS analysis and cell sorting, or cryopreserved as described for later use (23).

Isolation of human CD3+ , CD4+ , and CD8+ T cells from bnx bone marrow

Human T cell subsets were isolated from the bnx/hu bone marrow samples by immunoselection as described (13, 16, 17, 23). Human CD4+ cells were isolated from samples using magnetic beads directly conjugated to a monoclonal anti-CD4 Ab (Dynal anti-CD4 Dynabeads; Dynabull, Auburn, CA), according to the manufacturer’s instructions. CD8+ T cells were then isolated from the fraction depleted of CD4+ T cells, using Dynabeads directly conjugated to a monoclonal anti-CD8 Ab (Dynal) and the same technique. The resulting sorted CD3+CD4+ and CD3+CD8+ subsets consistently had greater than 98% purity.

Stimulation of human T cells recovered from bnx/hu mice

To simulate physiological conditions, as previously described (22), human T cell populations recovered from bnx/hu mice were stimulated with beads conjugated to secondary rat anti-mouse (RAM) or goat-anti-mouse (GAM) Ab (Becton Dickinson), coated with anti-CD3 and anti-CD28 Abs. The RAM secondary Ab is indirectly bound to the magnetic bead via a DNA linker and, therefore, RAM magnetic beads can be removed from cells by incubating the cells in DNase, allowing the bead-free cells to be further analyzed by FACS. GAM magnetic beads do not possess a DNA linker and, therefore, subsequent FACS analysis with cells coated with GAM beads (GB) is not possible. Therefore, cells stimulated with GB were used for RNA preparation only. The cDNA was used as prepared from the OKT3 hybridoma (American Type Culture Collection) by Dengpeng Yao at Childrens Hospital of Los Angeles. The cDNA was purchased from PharMingen (San Diego, CA). An appropriate amount of each Ab was mixed with the beads to coat each bead with 200 fg of each Ab. Human PBL and human CD3+ cells recovered from the bone marrow of bnx/hu mice were stimulated for 24 h in 25 cm2 flasks at a concentration of 1 × 106 cells/ml of medium, using a ratio of two beads to one cell. Following the designated stimulation period, the coated beads were removed with DNase, which cleaves the oligonucleotide linker between the bead and the secondary Ab. To provide potent and nonspecific stimulation, groups of cells were incubated with PMA plus ionomycin (25 ng/ml and 1 ng/ml, respectively).

RT-PCR analysis of cytokine up-regulation in response to activation

RNA was isolated from stimulated and nonstimulated cells using RNA STAT-60 (Tel-Test, Friendswood, TX). Samples were quantitated using a spectrophotometer, and equal amounts of RNA from all samples were subjected to first strand cDNA synthesis using the Superscript Preamplification System (Life Technologies, Gaithersburg, MD). For experiments in which human T cells were not sorted into CD4+ and CD8- subsets, the amount of RNA contributed by human T cells in samples from bnx/hu mice was first calculated from FACS analysis, which determined the percentage of CD3+ human T cells present in the sample. The same amount of RNA was then used from human PBL controls for first strand synthesis. This precise quantitation was necessary because human T cells in bnx/hu mice were present at a lower frequency than the frequency of human T cells found in PBL. Therefore, equal amounts of RNA from bnx/hu mice and normal PBL samples would not have been representative of equivalent amounts of T cell RNA.

Following cDNA amplification from the standardized bnx/hu and PBL samples, or from the human T cells and their subsets re-isolated from the bnx bone marrow, PCR was performed for IL-2, IL-4, and β2-microglobulin (β2M; used as a loading control). Samples were loaded on 2% ethidium bromide-stained gels, transferred to nylon membrane, and probed with human-specific oligonucleotides as described (15, 17, 23). Signals on autoradiograms were quantitated using a densitometer, and the cytokine signal to the β2M signal ratio was determined for stimulated and unstimulated samples for each group. The signal generated in stimulated samples was then reflected as the fold increase of that cytokine over unstimulated controls. RT-PCR was also performed on stimulated and unstimulated samples from mice not transplanted with human cells, to ensure that the primers used were human specific.

The primers used for RT-PCR were as follows: 1) for β2M, primer A, 5'-CTG GCC CTA TCT CCT TTG TC-3' and primer B, 5'-GTC TCG ACG AAG CAT GCT CAC AT-3' and primer B, 5'-AGG TAA TCC ATC TGT TCA GA-3' (cDNA product = 366 bp); 2) for IL-2, primer A, 5'-ACT CAC CAG GAT GCT CAC AT-3' and primer B, 5'-AGG TAA TCC ATC TGT TCA GA-3' (cDNA product = 266 bp); 3) for IL-4, primer A, 5'-TTC CCC CCT GTC TTC TCT CT-3' and primer B, 5'-TTC CTG TCG AGC CTT TTC AG-3' (cDNA product = 317 bp); and 4) for IFN-γ, primer A, 5'-GCA TCG TTT TGG GTG CTC GTG GTT ACT GC-3' and primer B, 5'-GTC TTT TTT TTC CGG TTC GCT GTT CTT AG-3' (cDNA product = 427 bp).

Analysis of CD25, HLA-DR, and CD45RA/CD45RO expression on CD3+ cells and CD4+ and CD8+ subsets recovered from bnx bone marrow

Following isolation from the bone marrow, and after stimulation, human CD3+ cells were assessed for levels of expression of the early activation markers CD25 and HLA-DR (both FITC-labeled Abs from Becton Dickinson, Mountain View, CA). Cells were blocked, incubated with Ab for 15–30 min on ice, then analyzed by FACS using the CellQuest program (Becton Dickinson).

Three-color FACS analysis was performed on CD4+ and CD8- subsets using CD45RA-FITC, CD45RO-PE and either CD4-PerCP or CD8-PerCP (all from Becton Dickinson) to determine CD45RA/CD45RO expression on CD4+ and CD8+ T cells from bnx/hu mice and from PBL controls. The enumeration of CD45RA-FITC vs CD45RO-PE levels were made using regions of CD4+ or CD8- PerCP bright cells that fell within the lymphocyte gate.

Results

Analysis of CD25 and HLA-DR expression in stimulated and unstimulated human T cells recovered from bnx mouse bone marrow

CD34+ or CD34+/CD38- cells were isolated and determined to be devoid of mature T cells (Fig. 1A). The cells were then cotransplanted into immune-deficient bnx mice with IL-3-producing human marrow stromal cells, or with a combination of huIL-3- and huIL-7-producing stromal cells. It was determined that no peripheral expansion of mature, contaminating T cells had contaminated the stem cell transplant inoculum by harvesting several mice 1 mo post-transplantation. No human CD4+ or CD8+ T cells were present in the bone marrow of the mice at that point (Fig. 1B), although the human CD45+ cell engraftment ranged from 5 to 11% marrow replacement (data not shown). This data demonstrates that there was no contamination by mature human T cells, which would be expected to have expanded in a xenogeneic response by 4 wk after inoculation. No human CD4+/CD8- (double-negative) cells were detected in the marrow of the mice at the
FIGURE 1.  A. CD34⁺ cells isolated from human bone marrow using immunoselection were devoid of contaminating CD3⁺ T cells. Detection was performed using anti-CD34 (HPCA2) and anti-CD3 Abs and FACS analysis. Ungated cells from the transplant inoculum are shown. B. One month post-transplantation, no human T cells could be detected in the bone marrow of the mice. C. Experimental schema for activation of human T lymphocytes from bnx/hu mice and from human PBL controls ex vivo. Activation was monitored by measuring up-regulation of CD25 expression by FACS and by measuring up-regulation of IL-2 and IL-4 by RT-PCR.
The human T lymphocytes that had developed in their bone marrow of bnx/hu mice were harvested between 8 and 12 mo post-transplantation. Therefore, to accomplish the current studies, the transplanted marrow of the mice until 4–6 mo after stem cell transplantation (5). We have previously reported that T cell differentiation from human hematopoietic stem cells in athymic bnx mice is not a rapid process. We have previously reported that significant numbers of human T cells are not recovered from the marrow of the mice until 4–6 mo after stem cell transplantation (5). Therefore, to accomplish the current studies, the transplanted mice were harvested between 8 and 12 mo post-transplantation. The human T lymphocytes that had developed in their bone marrow were collected and analyzed for phenotype and responsiveness to stimuli (Fig. 1C).

To determine whether human T cells within the bnx/hu bone marrow exist in an activated state, we tested the cells immediately upon recovery from the marrow for the activation markers CD25-PE and HLA-DR-FITC against the T cell marker, CD3-PE. FACS analysis of these cells revealed that 10.5 ± 0.5% of the human T cells were CD3+/CD25+ (Fig. 2). HLA-DR was also expressed at similar levels (9.7 ± 2.1% of the human T cells were CD3+/HLA-DR+). This data indicates that the majority of the human T cells exist in an unactivated state in the bone marrow of bnx mice, in contrast to the scid/hu PBL xenograft model, where >90% of the human T cells are HLA-DR+ (24, 25).

Impact of systemic IL-7 production on extrathymic human T cell development in bnx/hu mice

We next examined the influence of the systemic production of huIL-7 from genetically engineered stromal cells (mesenchymal stem cells) on the phenotypes and activation status of the human cells in the bnx bone marrow. Production of huIL-3 in bnx mice is necessary to sustain the human hematopoietic cell graft (5, 7). In bnx mice cotransplanted with human progenitors and stromal cells engineered to produce huIL-3, both T cells and myeloid cells developed, as we have described (5). In mice cotransplanted with human hematopoietic progenitor cells and human stromal cells engineered to secrete huIL-3 and huIL-7, only human T cells developed with a failure of mature human myeloid cell development. Human B cells were not found in any of the bnx/hu mice analyzed in these experiments, but methods have been developed more recently that allow B cell differentiation in bnx mice (19). While the myeloid graft was lost, systemic production of huIL-7 in the mice significantly increased the levels of human CD3+/CD4+ T cells that developed in the bnx bone marrow, and the proportion of the human graft that was comprised of CD3+/CD4+ cells (Table I). Although there was variability in the human CD3+/CD4+ vs CD3+/CD8+ levels from mouse to mouse, the presence of IL-7 in addition to IL-3 (n = 6) significantly increased the number of human CD4+ cells, as compared with IL-3 alone (n = 8, p < 0.05; Table I).

We next determined whether human T cells recovered from bnx/hu mice were responsive to activation stimuli in vitro, and whether the responsiveness could be influenced by the presence of huIL-7 in the bnx serum. Human T cells isolated from the bone marrow of both types of mice (cotransplanted with huIL-3 stroma alone (group 1, Table I) or a combination of huIL-3 plus huIL-7 stromas (group 2, Table I)) were stimulated in vitro for 24 h using CD3 and CD28 cross-linking or PMA plus ionomycin. A flowchart illustrating these experiments is shown in Fig. 1C. Briefly, CD34+ hematopoietic stem cells or CD34+ progenitor cells

Table I. Human T lymphocyte engraftment levels in the bone marrow of bnx mice 8–12 mo post-transplantation

<table>
<thead>
<tr>
<th>Mouse No.</th>
<th>Systemic Human Cytokines Produced</th>
<th>% Human CD45+ Cells in Bone Marrow</th>
<th>Proportion of CD3+/CD4+</th>
<th>Proportion of CD3+/CD8+</th>
<th>Proportion of CD3+</th>
<th>p Value, IL-3 vs IL-3 + IL-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
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<td></td>
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<td>32.6 ± 2.8</td>
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<tr>
<td>9</td>
<td>huIL-3 + huIL-7</td>
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<td>48.2</td>
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</tr>
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<td>31.3</td>
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<td>48.0</td>
<td>48.2</td>
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<tr>
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<td>56.1</td>
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<tr>
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<td>3.9</td>
<td>45.6</td>
<td>40.2</td>
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<td></td>
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<tr>
<td>Average ± SEM</td>
<td></td>
<td>4.5 ± 0.5</td>
<td>40 ± 3.3</td>
<td>49.6 ± 2.6</td>
<td>0.9 ± 0.5</td>
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</tr>
<tr>
<td>p Value, IL-3 vs IL-3 + IL-7</td>
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<td>0.69</td>
<td>0.004</td>
<td>0.78</td>
<td>6.1 × 10⁻⁷</td>
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</table>
isolated from human bone marrow were cotransplanted with huIL-3 plus huIL-7 stromas or with huIL-3 stroma alone into sublethally irradiated bnx mice via tail-vein injection as described (5, 7, 12–14, 16–18). The bone marrow from bnx/hu mice was harvested between 8 and 12 mo after transplantation, and the content of human cells of various lineages was determined by FACS analysis. Human T cells were isolated from the bnx/hu bone marrow samples, or whole bnx marrow was used in activation studies done using three different types of stimuli. Two different types of magnetic beads were used; one which was coated with a RAM secondary Ab and the other which was coated with a GAM secondary Ab (Fig. 1). Both types of bead were coated with anti-CD3 and anti-CD28 Abs, a method which has previously been shown to strongly stimulate T cells (22). T cells from bnx/hu bone marrow were stimulated for 24 h. At the end of the culture period, FACS analysis for CD25 and HLA-DR expression and RT-PCR for IL-2 and IL-4 were performed on stimulated and unstimulated cells.

The results from FACS analysis for CD25 expression on stimulated human PBL and bnx/hu T cells is shown in Fig. 3. Control T cells isolated from human peripheral blood showed an increase in CD25 expression in response to both anti-CD3 plus anti-CD28-coated magnetic beads (40.9 ± 5.6%) and to PMA plus ionomycin (79.1 ± 12.3%), in response to medium alone (2.8 ± 4.4%, Fig. 3). In contrast, human T cells recovered from the bone marrow of bnx mice from group 1 (Table I, cotransplanted with human hematopoietic progenitors and huIL-3 stroma (n = 4)), were hyporesponsive to both Ab-coated beads and to PMA plus ionomycin. Human T cells recovered from these mice showed little increase in CD25+CD3+ cells in response to anti-CD3 plus anti-CD28-coated beads, as compared with unstimulated controls (5.6 ± 4.4% vs 4.5 ± 5.6%, respectively; Fig. 3). The systemic production of huIL-7 in mice transplanted with the same donor stem cells (group 2, Table I) did not significantly increase the response to the stimulus of Ab-coated magnetic beads, as compared with unstimulated controls (7.9 ± 7.1% vs 3.2 ± 4.1%, respectively; Fig. 3). However, CD3+ human T cells that had developed in bnx/hu mice with systemic huIL-7 production (n = 3) showed a moderate increase in CD25 expression in response to PMA plus ionomycin, as compared with CD3+ cells from mice without systemic IL-7 (n = 4) (27.7 ± 7.3% vs 20.8 ± 13.3%, respectively; Fig. 3). This data indicated that systemic production of huIL-7 in the mice could have an effect on human T cell development, activation status, or mRNA stability.

RT-PCR for IL-2 and IL-4 mRNA in human T cells from bnx/hu mice vs PBL

We next wished to determine whether stimulation would lead to up-regulation of cytokine expression in human T cells recovered from the bnx bone marrow. Several mice from group 1 were selected for the initial studies. These mice had higher levels of CD8+ than CD4+ cells, as is typical in the bnx/hu system (5, 7, 12, 14, 16, 18). RT-PCR analysis of bone marrow cells from bnx/hu mice (n = 5) incubated for 30 h with anti-CD3 plus anti-CD28-coated magnetic beads showed markedly lower levels of IL-2 expression than normal human PBL controls (Fig. 4). The Ab-coated GB proved to be a stronger stimulus than the RAM beads (RB) coated with the same Abs, for both bnx/hu T cells and for human PBL T cells. As a result, a signal for IL-2 was not detected in human T cells from bnx/hu mice incubated with Ab-coated RB, but could be weakly detected when bone marrow cells from the same bnx/hu mice were incubated with Ab-coated GB. In addition, the amount of IL-2 up-regulation in human T cells from bnx/hu mice was significantly lower than that seen in human PBL controls when stimulated with anti-CD3 plus anti-CD28-coated GB (3- and 19-fold increase in IL-2 expression, respectively; Fig. 4). When bone marrow cells from the same bnx/hu mice were incubated in PMA plus ionomycin, the increase in IL-2 expression was significantly greater than the increase in IL-2 obtained with the Ab-coated GB. However, up-regulation of IL-2 in human T cells from bnx/hu mice in response to PMA plus ionomycin was significantly lower than that obtained in normal PBL controls in response to PMA plus ionomycin (12- and 30-fold increase in IL-2 expression, respectively; Fig. 4).

We also performed RT-PCR for IL-4 on cDNA prepared from the bnx/hu bone marrow samples described above. The human T cells were profoundly impaired in their ability to express IL-4 in response to all three activation stimuli (Fig. 4). Surprisingly, even incubating the bnx/hu T cells in PMA plus ionomycin did not result in an induction of IL-4 expression, although a strong up-regulation of IL-2 mRNA had been detected in the same cDNA samples (Fig. 4). The capacity to produce low levels of IL-2, but not IL-4, suggests that the majority of the bnx/hu T cells had been polarized in the mice toward a Th1 phenotype. The fact that murine IL-12, but not IL-4, is cross-reactive to human cells might have played a role in this aspect of the human T cell development.

Analysis of the influence of huIL-7 and levels of human CD4 vs CD8 T cells in the bnx bone marrow on the subsequent activation status

We next asked whether the reduced IL-2 and IL-4 expression observed in human T cells from the group 1 bnx/hu mice after activation may be due, in part, to the higher levels of CD8+ than CD4+ T cells. To answer this question, we performed the same...
FIGURE 4. IL-2 and IL-4 expression in human T cells from bnx/hu mice and from PBL controls in response to activating stimuli. A. Southern blot of IL-2 RT-PCR products from human peripheral blood control T cells and from human T cells recovered from two bnx/hu mice from group 1. B. Southern blot of IL-4 RT-PCR products generated using the same cDNA as was used for IL-2 RT-PCR in A. PBL, Peripheral blood; NTM, nontransplanted mouse; M, media alone; RB, RAM magnetic beads coated with anti-CD3 plus anti-CD28 Abs; GB, GAM magnetic beads coated with anti-CD3 plus anti-CD28 Abs; P + I, PMA plus ionomycin. C. Densitometry of the Southern blots for IL-2 and IL-4 RT-PCR products revealed that human T cells from group 1 bnx/hu mice were profoundly impaired in their ability to up-regulate IL-4 in response to all three activating stimuli.
experiments on samples from bnx/hu mice that had systemic huIL-7 production and relatively equal numbers of CD4+ and CD8+ cells (group 2). RT-PCR for IL-4 revealed that up-regulation of IL-4 was still significantly impaired in response to all three activation stimuli, as compared with human PBL controls (Fig. 5). PBL controls exhibited a 3.5-fold increase in IL-4 mRNA when stimulated with anti-CD3 plus anti-CD28-coated RB while human T cells from bnx/hu bone marrow showed no increase in IL-4 mRNA in response to Ab-coated RB. The combination of PMA plus ionomycin produced a slight, but not significant, increase in IL-4 mRNA in human T cells from bnx/hu mice, but this increase was significantly lower than normal PBL controls (1.3- to 3.3-fold increase in bnx/hu T cells vs a 16-fold increase in IL-4 mRNA from PBL). There was also a slight increase in IL-4 mRNA when human T cells from bnx/hu mice were stimulated with anti-CD3 plus anti-CD28-coated GB, but again, this increase was much lower than the response observed from human PBL controls (4- to 10-fold increase in bnx/hu T cells vs a 31 times increase in IL-4 mRNA for PBL). However, the human CD3+ samples isolated from the mice that had systemic IL-7 and equivalent CD4+ to CD8+ ratios (group 2) provided the only instance of IL-4 production that has been observed in bnx/hu T cells to date.

We also tested the capacity of the bnx/hu T cells from both groups of mice to induce expression of IFN-γ in response to the same stimuli used for the analysis of IL-2 and IL-4 production. Efficient and reproducible induction of IFN-γ was achieved in group 1 and group 2 cells only following propidium iodide stimulation, and the levels produced by the human T cells in either group were equivalent (data not shown). After stimulation by the anti-CD3- and anti-CD28-conjugated beads, little expression of IFN-γ was detected in cells from either the IL-7-treated or nontreated mice. There were no statistical differences in the responses from the group 1 and the group 2 mice. This data indicates that the IL-7 treatment did not induce the capacity of the extrathymically developed human T cells to produce IFN-γ in response to a physiological stimulus.

Of great interest, RT-PCR for IL-2 revealed that human T cells from the group 2 bnx mice, that had systemic huIL-7 production and equal levels of CD4+ and CD8+ cells in their bone marrow, were able to up-regulate IL-2 to levels comparable to human PBL controls in response to both αCD3 plus αCD28-coated magnetic beads (GB and RB) and to PMA plus ionomycin (Fig. 5). This data is in dramatic contrast to the hyporesponsive state observed in mice that had no systemic huIL-7 and lower levels of CD3+/CD4+ than CD3+/CD8+ cells in their marrow (group 1). It was not known at this point whether the critical factor in causing the human T cells to be more responsive after recovery from the marrow of the group 2 mice was the presence of huIL-7, systemically produced by engineered stromal cells cotransplanted into the mice, or the equivalent CD3+/CD4+ and CD3+/CD8+ ratios in those mice. It was possible that the higher human CD8+ levels in the group 1 mice had a suppressing or masking effect on the human CD4+ cells that had developed in the same marrow compartment. Therefore, we separated each subpopulation before performing the activation studies, in the next series of experiments.

**Discussion**

The bnx/hu model of human hematopoiesis provides a unique opportunity to study aspects of extrathymic human T lymphocyte development in an in vivo system. The bnx mouse lacks a thymus,
yet mature human CD3\(^+\)/CD4\(^+\), CD3\(^+\)/CD8\(^-\), αβ TCR-positive T lymphocytes develop within their bone marrow following transplantation with purified human CD34\(^+\) or CD34\(^+\)/CD38\(^-\) progenitors that are devoid of mature, contaminating T cells. In addition, immature double-positive CD4\(^+\)/CD8\(^-\) human T cells are also recovered from bnx/hu bone marrow. The recovery of both human myeloid and T lymphoid cells that had arisen from one common stem cell, as detected by single-cell clonal integration analysis.

**FIGURE 5.** IL-2 and IL-4 expression in T cells from bnx/hu mice with systemic huIL-7 production, possessing equal numbers of human CD4\(^+\) and CD8\(^-\) T cells, in response to activating stimuli. The symbols have the same definitions as those described in Fig. 4. A. Southern blot of IL-2-RT-PCR products from human PBL control T cells and from human T cells recovered from two bnx/hu mice from group 2. B. Southern blot of IL-4 RT-PCR products generated using the same cDNA as was used for IL-2 RT-PCR described in A. C. Densitometry of Southern blots for the IL-2 and IL-4 RT PCR products shown in Fig. 5, A and B. IL-4 expression is still dramatically reduced in human T cells from bnx/hu mice possessing systemic huIL-7, but IL-2 production is restored to normal levels.
is good evidence that the T cells had developed within the athymic mice, rather than having arisen from peripheral expansion of mature, contaminating T cells in the donor stem cell population.

In addition to the bnx/hu mice discussed in the present study, several other murine/human xenograft models have been developed to study the human immune system. Murine xenograft models of the human immune system are potentially quite valuable in determining the efficacy of vaccines, anti-HIV therapies, transplantation of xenogeneic organs, treatment of autoimmune disease, and acceleration of immune recovery after transplantation. *Scid*/*hu thy/liv* mice are created by implantation of human fetal liver next to fetal thymus from the same donor. In this system, there is continued production (5–11 mo) of human T cells from fetal liver, which have undergone selection in an implanted fragment of human fetal thymus (30, 31). The human T cells do not cause graft-vs-host disease (GVHD), perhaps because they recognize the murine tissue Ags as “self,” since murine dendritic cells colonize the human

FIGURE 6. IL-2 expression in sorted CD4$^+$ and CD8$^+$ human T cells from peripheral blood controls and from bnx/hu mice from group 1 in response to anti-CD3 plus anti-CD28-coated magnetic beads. A, FACS analysis of sorted CD4$^+$ and CD8$^+$ human T cells from peripheral blood showing that each subset isolated by positive selection was 98% pure. B, Southern blot of IL-2 RT-PCR products from sorted CD4$^+$ human T cells from peripheral blood and from bnx/hu mice from group 1. M, Media alone; GB, GAM magnetic beads coated with anti-CD3 plus anti-CD28 Abs. C, Southern blot of IL-2 RT-PCR products from sorted CD8$^+$ human T cells from peripheral blood and from bnx/hu mice from Group 1. D, Densitometry of Southern blot for IL-2 PCR products.
thymic implant. Human CD3+ cells recovered from scid/hu thy/liv mice can be readily stimulated by mitogens or anti-CD3 Abs, suggesting tolerance in vivo rather than anergy (30). In the hu-PBL-SCID model, SCID mice are reconstituted with human peripheral blood cells, containing T cells that have undergone the normal thymic selection process in the human donor (24, 25, 29, 30). All of the blood cells, containing T cells that have undergone the normal thymic selection process in the human donor (24, 25, 29, 30). Of interest, in mice that had been treated with huIL-7 at least partially restored the capacity of the extrathymic developing human T cells to undergo thymic selection through the TCR to become memory cells.

Human T cells are recovered from the bone marrow of bnx mice in the greatest numbers after 6 mo post-transplantation, suggesting a relatively slow and inefficient mechanism of differentiation. Studies have shown that T lymphocyte development occurs from stem cells by extrathymic mechanisms only in the absence of mature T cells (8–10, 32). Strober and colleagues (8–10) showed that mature T cells must be depleted from the transplant inoculum to allow extrathymic differentiation of new T cells in the marrow of athymic and euthymic murine transplant recipients. Tanzer and colleagues (32) demonstrated that the presence of T cells prevented T cell development from human hematopoietic progenitors in an in vitro system. In the bnx/hu model, purified CD34+ or CD34+/CD38− progenitors that are devoid of mature T cells are transplanted. Therefore, development of human T cells from stem cells in the bnx/hu system, devoid of mature T cells, agrees with the data from Tanzer’s and Strober’s groups and the principles of homeostasis.

Clues to the possible mechanisms for a type of “selection” of phenotypically mature T cells from human precursors that have successfully rearranged a TCR in the marrow of bnx mice come from the groups headed by Sykes and by Raulet (33, 34). Raulet’s group determined that reactivity to MHC proteins is inherent in randomly arranged TCRs, with or without positive and negative selection (33). Sykes’ group demonstrated for the first time that murine MHC molecules can positively select T cells from a widely disparate xenogeneic species, the pig (34). Therefore, the human T cells that arise from progenitors in bnx mice may undergo a type of positive selection in the murine recipient. The restriction of the TCRβ usage in human T cells that develop in bnx mice (independent of donor MHC), suggests positive selection of a limited number of clones by the murine microenvironment (5). In agreement with our observations, the human T cells in hu-PBL-SCID mice, generated by transplantation of mature human peripheral blood cells, initially display a varied TCRβ repertoire, which is narrowed over time to xenoreactive (but anergic) clones (24). In contrast, in scid/hu thy-liv mice, a varied TCRβ repertoire is present and is maintained in the human T cells that were selected by the implanted human fetal thymic tissue. The variations in the

Table II. Percentages of human CD4+ and CD8+ T cells recovered from the marrow of athymic bnx mice that are CD45RA+CD45RO− (naive) vs CD45RA−CD45RO+ (memory cells)

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Sample Type</th>
<th>RA+/RO−</th>
<th>RA−RO+</th>
<th>Sample Type</th>
<th>RA+/RO−</th>
<th>RA−RO+</th>
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<tr>
<td>Group 1-A</td>
<td>CD4+</td>
<td>66.4</td>
<td>33.5</td>
<td>CD8+</td>
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<td>Group 1-B</td>
<td>CD4+</td>
<td>70.8</td>
<td>29.1</td>
<td>CD8+</td>
<td>65.3</td>
<td>34.7</td>
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<td>Group 1-C</td>
<td>CD4+</td>
<td>74.9</td>
<td>25</td>
<td>CD8+</td>
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<td>Group 1-D</td>
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<td>69.6</td>
<td>30.4</td>
<td>CD8+</td>
<td>79.7</td>
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<td>CD8+</td>
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<td>27.1</td>
<td>CD8+</td>
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<tr>
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<td>32.1</td>
<td>70.1</td>
<td>29.9</td>
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</tr>
<tr>
<td>p value</td>
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<td>4.1 × 10−7</td>
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<tr>
<td>Group 2-A</td>
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<td>27.6</td>
<td>CD8+</td>
<td>59.9</td>
<td>40.1</td>
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<tr>
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<td>37.3</td>
<td>CD8+</td>
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<td>CD8+</td>
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<td>CD8+</td>
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<td>46.2</td>
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<td>Average ± SEM</td>
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<td>CD8+</td>
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<td>59.5</td>
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<td>CD8+</td>
<td>38.8</td>
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</tr>
<tr>
<td>Average ± SEM</td>
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<td>62.9</td>
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<td>58.8</td>
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</tr>
<tr>
<td>p value</td>
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TCRVβ repertoires highlight the differences between systems that contain human T cells that develop in murine systems in the presence and absence of human thymic selection.

In bnx/hu mice, although phenotypically mature human T cells are present, there have seldom been signs of GVHD (5). A potential cause for the lack of GVHD in bnx/hu mice could be that the human T cells are in a hyporesponsive state while in the bnx bone marrow, and cannot be easily stimulated upon removal from the animals. The current studies confirm this hypothesis. A similar situation has been seen in the hu-PBL-SCID system, where human T cells recultured from the mice cannot respond to stimulation for as long as 15–40 days (24). Tary-Lehman et al. (25) proposed that in the hu-PBL-SCID model, the continuous stimulation of anti-mouse reactive human T cells leads to the exhaustion of the response, resulting in peripheral tolerance or anergy. However, the factors causing the hyporesponsive state of the human T cells in the two xenograft systems cannot directly correspond to one another, because human T cells develop in bnx mice while exposed to murine histocompatibility molecules, in contrast to the hu-PBL-SCID T cells, which had developed in a human before transplantation into the mice.

In the present study, we examined the activation state of human T cells recovered from the bone marrow of immuno-deficient bnx/hu mice. We determined that there were low levels of CD25 (α-chain of the IL-2R) and HLA-DR (class II MHC) expression immediately after recovery. These results are in contrast to findings reported for human T cells recovered from hu-PBL-SCID mice (24). Although the human T cells recovered from hu-PBL-SCID chimeras were single positive (CD4+ or CD8+) and expressed the TCRαβ, they were HLA-DR+, in contrast to the bnx/hu T cells, which are 90% HLA-DR+. The authors hypothesized that the mature human T cells present in the initial inoculum were stimulated by murine MHC and costimulatory molecules, causing their expansion and activation. Our studies differ from those done in the hu-PBL-SCID system, in that the original inoculum in the bnx/hu system is devoid of contaminating mature human T cells. The human T cells that had developed from purified HSC in bnx mice also became anergic, as discussed further below. However, the bnx/hu T cells lacked significant expression of HLA-DR, in contrast to the human T cells recovered from scid/hu/PBL mice. This data indicates that the human T cells that developed in the bnx/hu bone marrow were not sufficiently stimulated by the murine microenvironment to up-regulate class II MHC expression.

To further study their state of responsiveness, human T cells recovered from the bnx/hu bone marrow were subjected to activation stimuli in vitro using magnetic beads coated with αCD3 and αCD28 Abs or a combination of PMA plus ionomycin. Subdividing the activation responses of the bnx/hu T cells by directly isolating human CD4+ and CD8+ T cells from the bnx/hu bone marrow were examined in comparison to human PBL controls. Sorted CD4+ and CD8+ T cells from human PBL produced high levels of IL-2, and the levels produced by the CD8+ cells were lower than those obtained in the CD4+ cell populations. This data was in agreement with a previous report demonstrating that in both cord blood T cells and adult blood T cells, IL-2 is produced predominantly by the CD4+ subset, as compared with the CD8+ subset (26). In the separated T cell subsets from the bnx/hu bone marrow, only CD4+ human T cells, but not CD8+ cells, produced low but detectable levels of IL-2. Of interest, when huIL-7 was supplied to the mice via engineered stromal cells, the IL-2, but not the IFN-γ or IL-4 induction in response to a physiological signal was normalized to the levels found in PBL controls. These data may be at least partially due to the fact that cytokine mRNA levels in T cells can be stabilized by IL-7 (35). If the IL-7 caused stabilization of IL-2 in the first CD4+ cells to develop, and thus increased the secretion of human IL-2, increased development or survival of higher levels of human CD4+ cells could result.

The profound impairment of IL-4 up-regulation in human T cells from bnx/hu mice in response to strong activating stimuli such as PMA plus ionomycin are similar to results found using cord blood T cells. Specifically, cord blood T cells produced less IL-2, IFN-γ, and TNF-α than adult T cells (26). Also, cord blood T cells did not up-regulate IL-4 in response to plate-bound αCD3 or to PMA plus ionomycin (27). Finally, PHA-stimulated PBMC from neonates and children under 10 produced significantly less IL-4 as compared with adults, and IL-4 production increased progressively with age (28). Cord blood T cells represent a population comprised primarily of naive, CD45RA+ T cells. However, adult blood T cells are comprised primarily of memory CD45RO+ T cells due to high exposure to different Ags. Therefore, we determined whether human T cells in the bnx/hu mice exist primarily in a naive, CD45RA+ state, which could at least partially explain their inability to up-regulate IL-4 in response to activating stimuli, similar to the situation found in naive cord blood T cells.

Since bnx/hu mice harvested more than 6 mo post-transplantation have no detectable CD34+ cells (36) and few human cells in the periphery, we had hypothesized that human hematopoiesis had slowed and that the T cell compartment would be composed primarily of memory cells. To our surprise, three-color FACS analysis for CD45RA/CD45RO expression on CD4+ and CD8+ human T cells showed that the majority of the bnx/hu mice analyzed had two to three times more CD45RA+ cells than CD45RO+ cells. Therefore, the human T cells appear to have developed and expanded without recent activation through the TCR to become CD45RO+. Similar observations have been made by the groups headed by Akbar and Taylor (37, 38).

The naive state of the human T cells in the bnx/hu mice may explain the lack of IL-4 production in these cells. The low IL-2 production by human T cells in sorted CD4+ and CD8+ subsets from bnx/hu mice could also be influenced by the naive state of the T cells. In cord blood, there is a lower percentage of CD4+CD45RA+ cells expressing IL-2 compared with CD4+CD45RO+ cells in adult blood. Also, the number of CD8+CD45RA+ and CD8+CD45RO+ cells producing IL-2 is lower in cord blood than adult blood (26).

In the majority of bnx/hu mice that have been tested, the levels of CD8+ T cells exceeded levels of CD4+ T cells (5, 7, 12, 14, 16–18, 22). However, in the majority of the mice that had been cotransplanted with human stromal cells engineered to secrete huIL-7 there were equivalent levels of human CD4+ and CD8+ cells (Table I). This data not only suggests that IL-7 can increase the ratio of CD4+ to CD8+ extrathymically derived human T cells (at least in the bnx/hu system), but gave us an opportunity to study the activation of these very interesting cells. RT-PCR for IL-2 in human T cells isolated from bnx/hu mice showed that when systemic huIL-7 was produced, and equal numbers of CD4+ and CD8+ human T cells were present, IL-2 production was comparable to the levels of IL-2 produced by normal PBL controls in response to both anti-CD3 plus anti-CD28-coated magnetic beads as well as to PMA plus ionomycin. This data was in sharp contrast to the extreme hyporesponsiveness of the human T cells recovered from mice that had no huIL-7, and had higher levels of CD8+ than CD4+ T cells. The non-IL-7-treated mice, engrafted with hyporesponsive T cells of primarily the CD8+ phenotype, are reminiscent of human BMT recipients with poor T cell responses (1–4, 26–28, 40). Human T cells recovered from the IL-7-treated mice demonstrated equivalent CD4 to CD8 ratios, higher levels of T cells with the “memory” phenotype, and had better responses to stimulation. Our data supports the theory that post-transplantation IL-7 therapy
could help to restore immune function post-transplantation (41, 42). To our knowledge, the current studies describe the first murine xenograft model in which extrathymic human T cell development can be studied in the absence of residual, mature T cells, which can cloud the results via peripheral expansion (1, 4).

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References


