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Regulation of Human Cell Engraftment and Development of EBV-Related Lymphoproliferative Disorders in Hu-PBL-scid Mice

Eric J. Wagar,* Mandy A. Cromwell,† Leonard D. Shultz,§ Bruce A. Woda,§ John L. Sullivan,‡ RuthAnn M. Hesselton,‡ and Dale L. Greiner‡*

Human PBMC engraft in mice homozygous for the severe combined immunodeficiency (Prkdc<sup>scid</sup>) mutation (Hu-PBL-scid mice). Hu-PBL-NOD-scid mice generate 5- to 10-fold higher levels of human cells than do Hu-PBL-C.B-17-scid mice, and Hu-PBL-NOD-scid β<sub>2</sub>-microglobulin-null (NOD-scid-B2m<sup>null</sup>) mice support even higher levels of engraftment, particularly CD4<sup>+</sup> T cells. The basis for increased engraftment of human PBMC and the functional capabilities of these cells in NOD-scid and NOD-scid-B2m<sup>null</sup> mice are unknown. We now report that human cell proliferation in NOD-scid mice increased after in vivo depletion of NK cells. Human cell engraftment depended on CD4<sup>+</sup> cells and required CD40-CD154 interaction, but engrafted CD4<sup>+</sup> cells rapidly became nonresponsive to anti-CD3 Ab stimulation. Depletion of human CD8<sup>+</sup> cells led to increased human CD4<sup>+</sup> and CD20<sup>+</sup> cell engraftment and increased levels of human Ig. We further document that Hu-PBL-NOD-scid mice are resistant to development of human EBV-related lymphoproliferative disorders. These disorders, however, develop rapidly following depletion of human CD8<sup>+</sup> cells and are prevented by re-engraftment of CD8<sup>+</sup> T cells. These data demonstrate that 1) murine NK cells regulate human cell engraftment in scid recipients; 2) human CD4<sup>+</sup> cells are required for human CD8<sup>+</sup> cell engraftment; and 3) once engrafted, human CD8<sup>+</sup> cells regulate human CD4<sup>+</sup> and CD20<sup>+</sup> cell expansion. Ig levels, and outgrowth of EBV-related lymphoproliferative disorders. We propose that the Hu-PBL-NOD-scid model is suitable for the in vivo analysis of immunoregulatory interactions between human CD4<sup>+</sup> and CD8<sup>+</sup> cells. The Journal of Immunology, 2000, 165: 518–527.

A small animal model in which human lymphocytes, particularly human CD4<sup>+</sup> T cells, can be studied in an in vivo environment would be valuable for understanding human immune responses in vivo. Such a model would facilitate research on human tumor biology, transplantation, and autoimmunity and provide a small animal model for the study of human-specific infectious agents such as HIV. One potential model is the Hu-PBL-scid mouse first described >10 years ago (1). In this model, C.B-17-Prkdc<sup>scid</sup>/Prkdc<sup>scid</sup> (C.B-17-scid) mice are injected i.p. with human PBMC. Although technically easy to establish, the Hu-PBL-C.B-17-scid model is limited in its utility due to the relatively low levels of engraftment and the anergic state of human cells engrafted in the mice (2–4). Injection of larger numbers of human cells to increase levels of cell engraftment leads to development of EBV-related human lymphoproliferative disorders (LPD) in the majority of animals (5, 6).

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Abbreviations used in this paper: LPD, lymphoproliferative disorders; NOD-scid-B2m<sup>null</sup>; NOD-scid β<sub>2</sub>-microglobulin-null; B-LCL, B-lymphoblastoid cells; CFSE, 5-(and-6)-carboxyfluorescein succinimidyl esters; EBER, EBV-related early antigen RNA; MST, median survival time.

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immunoregulatory interactions between human CD4+ and CD8+ cells, and that human CD8+ cells regulate the in vivo expression of EBV-related LPD.

Materials and Methods

Animals

NOD/LtSz-SJL-Pkdnull (NOD-scid), NOD/LtSz-SJL-Pkdnull/Pkdnull (NOD-scid-B2mnull), and C57-129/Pkdnull/Pkdnull (C-B17-scid) mice were obtained from colonies maintained by LDS at The Jackson Laboratory (Bar Harbor, ME). All animals were certified to be free of scid mice, 2m null mice were obtained from colonies maintained by LDS at The Jackson Laboratory (Bar Harbor, ME). All animals were certified to be free of EBV-related LPD development, 50–60% of mice obtained from a single donor. The percentage and number of total CD4+ and CD8+ cells in the spleen and peritoneal cavity and the percentages of the CD4+ and CD8+ cell subsets that express each activation Ag are shown.

Table 1. Activation phenotype of human CD4+ and CD8+ cells in Hu-PBL-NOD-scid and Hu-PBL-NOD-scid-B2mnull mice

<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Day 7</td>
<td>Day 14</td>
<td>Day 21</td>
<td>Day 7</td>
</tr>
<tr>
<td>Total CD4+ (% (x10^6)</td>
<td>13.2</td>
<td>21.8 ± 10.7</td>
<td>14.2 ± 5.5</td>
<td>13.2</td>
</tr>
<tr>
<td>% CD25+ CD4+</td>
<td>39.8</td>
<td>27.4 ± 3.3</td>
<td>15.1 ± 1.1</td>
<td>37.7</td>
</tr>
<tr>
<td>% CD45R0+ CD4+</td>
<td>55.4</td>
<td>92.8 ± 4.4</td>
<td>93.7 ± 3.6</td>
<td>44.8</td>
</tr>
<tr>
<td>% HLA-DR CD4+</td>
<td>32.9</td>
<td>44.4 ± 17.7</td>
<td>57.9 ± 0.8</td>
<td>28.0</td>
</tr>
<tr>
<td>Total CD4+ (%) (x10^6)</td>
<td>16.9</td>
<td>30.3 ± 8.8</td>
<td>17.8 ± 11.6</td>
<td>7.2</td>
</tr>
<tr>
<td>% CD25+ CD8+</td>
<td>13.8</td>
<td>7.6 ± 7.1</td>
<td>1.3 ± 0.7</td>
<td>19.4</td>
</tr>
<tr>
<td>% CD45R0+ CD8+</td>
<td>63.4</td>
<td>91.8 ± 4.2</td>
<td>69.8 ± 11.4</td>
<td>60.0</td>
</tr>
<tr>
<td>% HLA-DR CD8+</td>
<td>65.9</td>
<td>89.7 ± 2.9</td>
<td>80.4 ± 8.3</td>
<td>54.0</td>
</tr>
<tr>
<td>CD4:CD8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.9</td>
<td>1.8</td>
</tr>
</tbody>
</table>

* Peritoneal cavity and spleen cells were recovered from Hu-PBL-NOD-scid and Hu-PBL-NOD-scid-B2mnull mice 1, 2, and 3 wk after the i.p. injection of 20 x 10^6 human PBMC obtained from a single donor. The percentage and total number of CD4+ and CD8+ cells in the spleen and peritoneal cavity and the percentages of the CD4+ and CD8+ cell subsets that express each activation Ag are shown.

This value represents a pool of five individual mice.

† At day 14, the value represents a single mouse that displayed engraftment of human cells in the spleen that was >1% in a group of five mice. Similar data were obtained using a different donor.

Cell preparations

Splenic single cell suspensions were prepared from Hu-PBL-scid mice by teasing spleens from frosted microscope slides. RBC were lysed in hypotonic ammonium chloride lysis buffer and rinsed twice in PBS supplemented with 5% FBS (HyClone, Logan UT), and the number of viable cells was determined by exclusion of trypan blue using a hemocytometer. Peritoneal cavity mononuclear cells were recovered from Hu-PBL-scid mice at necropsy by lavaging the peritoneal cavity with 5 ml of RPMI 1640 warmed to 37°C.

Purified populations of human CD4+ and CD8+ peripheral blood T cells were prepared by positive selection using immunomagnetic beads (Dynabeads M-450, Dynal, Oslo, Norway). Cells were detached from the beads using the Detach-A-Bead reagent (Dynal) according to the manufacturer’s instructions.

Lymphoblastoid cell lines (B-LCL) were initiated from donor peripheral blood mononuclear cells by induction with supernatant from the EBV-producing cell line B95.8 as previously described (22). B-LCL lines were maintained in culture for use as targets or stimulators for EBV-specific T cell assays.

To generate EBV-specific or allo-specific CTLs, standard protocols were used. Briefly, NK-depleted PBMC were cultured in complete medium with mitomycin C-treated autologous or allogeneic B-LCLs, respectively. Cultures were restimulated weekly with mitomycin C-treated B-LCL and fed twice weekly with fresh complete medium containing 20 U/ml recombinant human IL-2. Cell lines were tested for phenotype and cytolytic activity after 3–4 wk in culture.

Relative cytotoxic activity of EBV-specific and allo-specific CTLs was determined by calculating lytic units based on cytolytic activity in a 51Cr release assay (23). The allo-specific CTLs were cytotoxic to allogeneic B-LCLs, but displayed no cytotoxic activity against autologous B-LCLs (data not shown).

Experimental treatments

Establishment of Hu-PBL-scid mice. Human PBMC were prepared from normal platelet-pheresis donors as previously described (18). Unless otherwise noted, mice were injected i.p. with 20 x 10^6 human PBMC in RPMI within 3 h of recovery following purification of human mononuclear cells by Ficoll-Hypaque density gradient centrifugation. In certain studies of EBV-related LPD development, 50–60 x 10^6 human PBMC were injected i.p. Analysis of engrafted mice was performed 4 wk after injection of PBMC unless otherwise noted. For ease of presentation, we have provided results from a single donor in Table 1. With the single exception of the histology photomicrograph representative of multiple animals from multiple donors, we showed pooled results from multiple donors in all other tables and figures.
Ab treatment. For analysis of the effects of in vivo Ab treatment on engraftment, Hu-PBL-scid mice were injected i.p. with 250 μg of anti-human CD4, anti-human CD8, or anti-human CD154 mAb twice weekly beginning on the day of human PBMC injection and continued to the conclusion of the experiment. Separate cohorts of Hu-PBL-NOD-scid mice were treated i.p. with 1.0 mg of anti-mouse CD122 mAb on the day of PBMC injection and once weekly to the conclusion of the experiment. In additional experiments of EBV-related LDLP development, a single injection of 250–500 μg of anti-human CD4, anti-human CD8, or anti-human CD16 mAb was given 1–3 days after engraftment of human PBMC. In all experiments the efficiency of cell subset depletion was assessed by flow cytometric analysis.

Histology and immunohistochemistry
Tissues were recovered from mice at necropsy, fixed in 10% buffered formalin, and embedded in paraffin. Sections 4 μm thick were cut. For routine histology, sections were stained with hematoxylin and eosin. The sections were interpreted by B.A.W. without knowledge of the experimental design. For immunohistochemistry, sections were heated at 65°C for 30 min, then deparaffinized and hydrated through a series of xylene and alcohol baths before staining. The slides were microwaved in a proprietary citrate-buff- ered Ag retrieval solution (BioTek Solutions, Santa Barbara, CA) for 5 min in an 800-W microwave oven. After replenishment of this solution the slides were microwaved for another 5 min, then allowed to cool for 20 min. Immunohistochemical staining was performed with mAb specific for human CD3, CD20, Igk, and Igλ chains using a standard avidin/biotin complex method as implemented on a Techmate 1000 (BioTek) automated immunostainer. The sections were counterstained with hematoxylin. To detect EBV early RNA (EBER) by in situ hybridization, sections were deparaffinized and washed in 2× sodium chloride/sodium citrate buffer. The sections were hybridized with a biotinylated EBER oligonucleotide probe at 37°C for 18 h. After washing in sodium chloride/sodium citrate buffer, the bound probe was detected using the avidin/biotin complex method.

Quantification of human Ig levels
Blood was collected from the retro-orbital plexus of individual engrafted mice, and levels of human Ig in sera were determined by nephelometry using a Beckman ARRAY 360 CD Serology System (Beckman Coulter, Fullerton, CA).

Mixed lymphocyte cultures
Murine stimulator cells were prepared by teasing spleens recovered from NOD-scid or NOD-scid-B2mnull mice between frosted microscope slides to obtain single-cell suspensions. RBC were lysed with hypotonic ammonium chloride lysis solution, and the nucleated cells were washed in RPMI supplemented with 10% FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.29 mg/ml l-glutamine (complete medium); counted; and sus- pended in complete medium at 8 × 10^6 cells/ml. The stimulator cells were then exposed to 2000 rad 137Cs radiation using a Gammacell 40 (Atomic Energy of Canada, Ottawa, Canada). Murine stimulator cells (8 × 10^6) in complete medium were added to each well of 96-well flat-bottom tissue culture plates (Falcon, Becton Dickinson Labware, Lincoln Park, NJ). Responder human PBMC (2.5 × 10^5 in 100-μl aliquots) were added to triplicate wells, and the mixture was cultured for 6 days at 37°C in a humidified atmosphere of 95% air in 5% CO₂. For the last 16 h of culture, the pulsed radioactivity was quantified as described above.

Flow cytometry
Spleen or peritoneal cavity cells recovered from Hu-PBL-scid mice were concentrated by centrifugation and washed in PBS plus 5% FBS, aliquoted into microtiter plates, and prepared for three-color flow cytometric analysis by incubation with PE-anti-murine-CD45, anti-human CD4 or anti-human CD8 conjugated to PE-Cy5, and FITC-conjugated anti-human CD25, anti-human CD45R0, and anti-human HLA-DR. Cells were incubated for the presence of 1 μg of Ab/10⁶ cells for 20 min at 4°C, washed three times in PBS plus 5% FBS, and fixed in 1% paraformaldehyde in PBS plus 0.02% NaN₃, for analysis.

At least 15,000 events were acquired on Becton Dickinson FACs equip- ment (Becton Dickinson, San Jose, CA), and the data were analyzed with WinMDI software (J. Trotter, The Scripps Research Institute, La Jolla, CA). In all analyses, murine CD45* cells labeled with anti-Ly5 mAb were excluded from further analysis. On the remaining cells, a two-color histo- gram of CD4 or CD8 vs an activation or proliferation marker (forward scatter, CD25, CD45R0, or HLA-DR) was used to determine the percentage of double-positive cells. Percentages are the mean ± SD of CD4+ or CD8+ cells that were also positive for the expression of an activation marker (CD25, CD45R0, or HLA-DR). The percentage of large cells was determined by increases in the forward light scattering properties compared with freshly isolated, nonstimulated CD4+ or CD8+ cells. Isotype Ab controls were used to assist in the determination of negative and positive populations.

Analysis of cell proliferation using 5-(and-6)-carboxyfluorescein succinimidyl ester (CFSE)-labeled cells
Human PBMC were labeled with CFSE (Molecular Probes, Eugene OR) as described previously (24). Labeled cells (20 × 10^6) were injected into the peritoneal cavity of NOD-scid mice, NOD-scid-B2mnull mice, and NOD- scid mice treated previously with anti-mouse CD122 mAb. The cells were recovered 6 days later by lavaging the peritoneal cavity with 5 ml of RPMI 1640 warmed to 37°C. Recovered cells were suspended in PBS plus 5% FBS, aliquoted into 96-well microtiter plates and labeled with anti-murine CD45-PE and either anti-human-CD4 or anti-human-CD8 mAb conjugated to PE-Cy5. Labeled cells were washed twice in PBS plus 5% FBS, fixed in 1% paraformaldehyde, and analyzed by flow cytometry.

Statistics
In experiments on development of EBV-related LDLP, p values were cal- culated using the method of Kaplan and Meier (25). The equality of survival time distributions for animals in different treatment groups was tested using the log rank statistic (25). For analysis of proliferation using CFSE dye labeling (Table II) and for analysis of human cells in the spleen of scid mice in Table IV, comparisons of means used one-way ANOVA (26) and the Bonferroni test for a posteriori contrasts (27). For comparison between multiple groups in the remaining experiments, we used the Bonferroni adjusted unpaired t test (27).

Results
Progression of human PBMC in Hu-PBL-NOD-scid and NOD- scid-B2mnull mice from naive to activated to anergic phenotype
Human PBLs in C.B-17-scid mice have been reported to have an activated phenotype, but are functionally anergic (28–30). To determine the kinetics of progression of engrafted human cells from that of a naive to an activated to an anergic phenotype, peritoneal cavity and spleen cells from separate cohorts of Hu-PBL-NOD- scid and Hu-PBL-NOD-scid-B2mnull mice established from a single PBMC donor were analyzed at 1, 2, and 3 wk after engraftment. The phenotype of peritoneal cavity cells 1 wk after engraftment resembled that of a naive to an activated to an anergic phenotype. The proportion of cells exhibiting characteristics of activated, CD25+ T cells increased with similar kinetics in both the peritoneal cavity and spleen. There was no evidence of tissue compartmentalization of naive or activated human cells between the peritoneal cavity and
spleen at 2 and 3 wk after engraftment, time points when direct comparisons were possible. The phenotype of human cells in the spleen at 4 wk was similar to that observed at 3 wk (data not shown).

To confirm that the activated CD25<sup>-</sup> human T cells at 4 wk after engraftment exhibited functional characteristics consistent with an anergic phenotype, we performed in vitro analyses. Human cells recovered from the spleens of Hu-PBL-NOD-<i>scid</i> and Hu-PBL-NOD-<i>scid</i>-<i>B<sub>2mnull</sub></i> mice that were incubated in the presence of plate-bound anti-human CD3 and anti-human CD28 mAb did not proliferate (Fig. 1). Addition of recombinant human IL-2, however, supported high levels of proliferation (Fig. 1). This functional characteristic is consistent with that of an anergic population described previously for human cells recovered from PBMC-engrafted C.B-17-<i>scid</i> mice (2).

Proliferation of human cells in the peritoneal cavity of NOD-<i>scid</i> and NOD-<i>scid</i>-<i>B<sub>2mnull</sub></i> mice

NOD-<i>scid</i>-<i>B<sub>2mnull</sub></i> mice engraft higher levels of human cells than do NOD-<i>scid</i> mice (20). This may result from differential expression of MHC class I on the murine stimulator cells, because the <i>B<sub>2mnull</sub></i> allele prevents MHC class I expression. To quantify the in vivo proliferative activity of human CD4<sup>+</sup> and CD8<sup>+</sup> cells, we employed the intravital dye CFSE. This dye labels lymphoid cells (24), and the uniform dilution of dye in cells as they divide can be used to determine whether labeled cells have undergone cell division (31).

Human PBMC labeled with CFSE were injected into the peritoneal cavity of NOD-<i>scid</i> and NOD-<i>scid</i>-<i>B<sub>2mnull</sub></i> mice and recovered 1 wk after engraftment for flow cytometric analysis. The percentage of CD8<sup>+</sup> CFSE-labeled cells that had undergone division in NOD-<i>scid</i>-<i>B<sub>2mnull</sub></i> mice was significantly greater than that observed in NOD-<i>scid</i> mice (Table II).

**Activation of human T cells by NOD-<i>scid</i> and NOD-<i>scid</i>-<i>B<sub>2mnull</sub></i> APC**

We next analyzed the activation of human cells by NOD-<i>scid</i> and NOD-<i>scid</i>-<i>B<sub>2mnull</sub></i> mice using in vitro xenogeneic mixed lymphocyte cultures. There were no detectable significant differences between the activation phenotype of human CD4<sup>+</sup> or CD8<sup>+</sup> T cells incubated with NOD-<i>scid</i> irradiated splenocytes compared with that observed following stimulation with NOD-<i>scid</i>-<i>B<sub>2mnull</sub></i> irradiated splenocytes (Table III). The CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets in both cultures exhibited a phenotypic pattern consistent with activation, including comparable increases in forward light-scattering properties and cell surface expression of CD25, CD45R0, and HLA-DR (Table III).

To determine the association between the activation phenotype of human PBMCs in the mixed lymphocyte cultures with human cell proliferation, we quantified [<sup>3</sup>H]thymidine incorporation on day 6 of culture. Unstimulated human PBMC incorporated 1,989 ± 1,043 cpm (n = 3; mean ± 1 SD). Human PBMC stimulated with NOD-<i>scid</i> (n = 3) or NOD-<i>scid</i>-<i>B<sub>2mnull</sub></i> (n = 3) irradiated splenocytes incorporated 18,087 ± 1,324 and 14,473 ± 4,626 cpm, respectively (p = NS). These data demonstrate that the absence of murine MHC class I on the surface of APC obtained from NOD-<i>scid</i>-<i>B<sub>2mnull</sub></i> mice does not have a significant effect on the in vitro proliferative response of human T cells.

**Human cell engraftment in NOD-<i>scid</i> mice is dependent on host NK cell activity**

We next investigated whether the increased engraftment of human cells in NOD-<i>scid</i>-<i>B<sub>2mnull</sub></i> mice was due to their severe deficiency in NK cell activity compared with the relatively less severe deficiency of NK cells in NOD-<i>scid</i> mice (20). To test this, murine NK cells were depleted in vivo in a cohort of NOD-<i>scid</i> mice using the anti-mouse CD122 mAb TM-β1 that recognizes the mouse IL-2R β-chain expressed on NK cells (32). At 4 wk after engraftment Hu-PBL-NOD-<i>scid</i> mice treated with anti-mouse CD122 mAb had significantly higher percentages of human cells in their spleens than did Hu-PBL-NOD-<i>scid</i> mice that did not receive TM-β1 mAb (Fig. 2). The absolute number of human cells in the spleen was also elevated, with an average of 18 ± 10<sup>6</sup> ± 4.5 ± 10<sup>6</sup> cells in

---

**Table II. Proliferation of CFSE-labeled human CD4<sup>+</sup> and CD8<sup>+</sup> cells in the peritoneal cavity of Hu-PBL-NOD-<i>scid</i> and Hu-PBL-NOD-<i>scid</i>-<i>B<sub>2mnull</sub></i> mice 7 days after injection**

<table>
<thead>
<tr>
<th>Recipient Treatment</th>
<th>Strain and Human CD4&lt;sup&gt;+&lt;/sup&gt; Cells That Have Undergone Cell Division (%)</th>
<th>Human CD8&lt;sup&gt;+&lt;/sup&gt; Cells That Have Undergone Cell Division (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOD-&lt;i&gt;scid&lt;/i&gt;</td>
<td>11.5 ± 5.5</td>
<td>30.0 ± 11.0</td>
</tr>
<tr>
<td>NOD-&lt;i&gt;scid&lt;/i&gt;-&lt;i&gt;B&lt;sub&gt;2mnull&lt;/sub&gt;&lt;/i&gt;</td>
<td>19.4 ± 9.7</td>
<td>47.3 ± 19.7*</td>
</tr>
<tr>
<td>NOD-&lt;i&gt;scid&lt;/i&gt; treated with anti-CD122 mAb</td>
<td>79.5 ± 10.4**</td>
<td>77.4 ± 9.5**</td>
</tr>
</tbody>
</table>

*Percentages of human CD4<sup>+</sup> and CD8<sup>+</sup> cells that had undergone cell division in the peritoneal cavity of individual animals was determined by flow cytometry. Values represent the mean percent ± SEM for two (NOD-<i>scid</i> mice, NOD-<i>scid</i>-<i>B<sub>2mnull</sub></i> mice) independent experiments.

†, p < 0.01 vs NOD-<i>scid</i>; *, p < 0.001 vs all other groups.

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**FIGURE 1.** In vitro stimulation of human cells recovered from the spleens of Hu-PBL-NOD-<i>scid</i> and Hu-PBL-NOD-<i>scid</i>-<i>B<sub>2mnull</sub></i> mice. NOD-<i>scid</i>-<i>B<sub>2mnull</sub></i> mice were injected i.p. with 20 × 10<sup>6</sup> human PBMC. At 4 wk spleen cell pools were prepared, and 2.5 × 10<sup>6</sup> cells/well were cultured in triplicate without stimulation (●) or in the presence of plate-bound anti-human CD3 plus anti-human CD28 mAb (○), recombinant human IL-2 (□), or both plate-bound anti-human CD3 plus anti-human CD28 mAb and IL-2 (◇). The cells were harvested on day 6 of culture following incubation for the last 16 h in the presence of 1 μCi of [<sup>3</sup>H]thymidine. Values are the mean ± SEM. *, Significantly different from unstimulated and Ab-stimulated cultures, p < 0.005.
anti-mouse CD122 mAb-treated hosts (mean ± SEM; n = 15) compared with $1.4 \times 10^6 \pm 0.8 \times 10^6$ cells in untreated hosts ($n = 19$; $p < 0.003$).

In addition to significantly higher numbers of human CD45$^+$ cells in the spleen, the ratio of CD4$^+$ to CD8$^+$ cells was significantly higher in mice treated with anti-mouse CD122 mAb (2.9 ± 0.6) than in untreated controls (0.7 ± 0.1; $p < 0.007$).

Treatment with TM-1 anti-mouse CD122 mAb also enhanced the proliferation of engrafted cells in NOD-scid mice by day 6 as measured by CFSE staining. The percentages of CD4$^+$ cells (79.5 ± 10.4%) and CD8$^+$ cells (77.4 ± 9.5%) that had undergone cell division in mice treated with TM-1 mAb was significantly higher than that observed in either NOD-scid or NOD-scid-B2mnull mice injected with PBMC from the same donor (Table II).

**CD4$^+$ cells and CD40-CD154 interactions are required for engraftment of human cells in scid mice**

The host innate immune system clearly has a critical role in the cell subset and number of human cells that engraft in Hu-PBL-scid mice. We next studied the roles of the human CD4$^+$ and CD8$^+$ T cell subsets in regulating human cell engraftment in NOD-scid and NOD-scid-B2mnull mice. To prevent human CD4$^+$ cell engraftment, we treated Hu-PBL-NOD-scid and Hu-PBL-NOD-scid-B2mnull mice with a depleting anti-human CD4 mAb. Although both CD4$^+$ and CD8$^+$ human cells readily engrafted in untreated recipients, specific depletion of only CD4$^+$ cells led to the ablation of both human CD4$^+$ and CD8$^+$ cell engraftment (Table IV). As shown previously (33), human CD4$^+$ and CD8$^+$ cell engraftment was also inhibited by treatment with anti-human CD154 mAb, a costimulatory molecule expressed predominately on activated CD4$^+$, but not CD8$^+$, T cells (Table IV).

**CD8$^+$ cells regulate CD4$^+$ cell expansion in Hu-PBL-scid mice**

To determine whether human CD8$^+$ cells are required for CD4$^+$ cell engraftment, we treated a cohort of Hu-PBL-NOD-scid and Hu-PBL-NOD-scid-B2mnull mice with a depleting anti-human CD8 mAb. Engraftment of total human cells in the spleen at 4 wk, as determined by expression of human CD45, was similar to that obtained in otherwise untreated Hu-PBL-NOD-scid and Hu-PBL-NOD-scid-B2mnull mice (Table IV). However, the majority of the engrafted T cells were CD4$^+$; few to no human CD8$^+$ cells were detected in the blood or spleen (Table IV).

Corresponding with increased engraftment of human CD4$^+$ cells in the spleen, the levels of human Ig in Hu-PBL-NOD-scid mice following treatment with anti-human CD8 mAb were significantly increased (497.5 ± 280.6 μg/ml IgG and 151.4 ± 81.6 μg/ml IgM; $n = 7$) compared with those observed in control Hu-PBL-NOD-scid mice (80.5 ± 33.7 μg/ml IgG ($p < 0.02$); 21.0 ± 9.0 μg/ml IgM ($p < 0.02$); $n = 13$).

Depletion of CD8$^+$ cells increases the development of EBV-related lymphoproliferative disorders in Hu-PBL-NOD-scid mice

Injection of high numbers of PBMC (≥50 × 10⁶) obtained from EBV-seropositive donors frequently leads to the outgrowth of EBV-related LPD in Hu-PBL-C.B-17-scid mice (6, 11). Despite increased levels in the engraftment of human cells in Hu-PBL-NOD-scid mice following the injection of 20 × 10⁶ human PBMC, we observed that few mice exhibited EBV-related LPD that were detectable by necropsy at 4–6 wk. To quantify this, we injected separate cohorts of NOD-scid and C.B-17-scid mice with 50–60 × 10⁶ PBMC obtained from two different EBV-seropositive donors. In Hu-PBL-NOD-scid mice, EBV-related LPD were observed at the 16 wk necropsy in 4 of 12 mice (33%) injected with PBMC from donor 1 and in 25 of 46 (54%) of mice injected with PBMC from donor 2.
The morphologic features of the tissues examined from the CD8-depleted animals were uniform. These animals (six of six; by $\chi^2$ test, $p < 0.01$ vs non-CD8-depleted animals) all shared detectable EBER-positive cells. In some animals only a few EBER positive cells were detectable, while in others many positive cells were noted (Fig. 3). Histologic examination showed a marked proliferation of large transformed lymphoid cells (Fig. 3). The infiltrates were quite polymorphous and were composed of a mixture of lymphoblasts, immunoblasts, and plasma cells. Germinal centers were never identified. Immunoperoxidase studies (Fig. 3, C and D) generally showed both CD3$^+$ T cells and CD20$^+$ B cells. In some tissues CD20$^+$ B cells and Igk$^+$ and Ig\(\lambda^+\) plasma cells were numerous.

Adaptive transfer of CD8$^+$ T cells into CD8-depleted Hu-PBL-NOD-scid mice restores resistance to EBV-related lymphoproliferative disorders

We next confirmed that human CD8$^+$ T cells were the primary mediators of resistance in vivo to the development of EBV-related LPD in Hu-PBL-NOD-scid mice. Purified populations of CD4$^+$ or CD8$^+$ human T cells were adoptively transferred into NOD-scid mice that had received 1) 50–60 × 10$^6$ PBMC from the same original donor and 2) a single injection of anti-CD8 mAb at 1–3 days after cell engraftment. Evidence for the development of EBV-related LPD was apparent at necropsy 16 wk later in 29 of 58 (50%) of the Hu-PBL-NOD-scid mice that received cells without coinjection of anti-CD8 Ab (Fig. 4). This rate was comparable to that observed in our initial study above. CD8-depleted Hu-PBL-NOD-scid mice uniformly (20 of 20, 100%) developed EBV-related LPD by 16 wk after cell engraftment (Fig. 4). In contrast, i.p. injection of 5–10 × 10$^6$ CD8$^+$ T cells from the original PBMC donor on days 16 and 34 after initial PBMC injection restored CD8$^+$ T cell engraftment and reduced the incidence of EBV-related LPD in CD8-depleted Hu-PBL-NOD-scid mice from 100% to only 1 of 10 (10%). The transfer of 5–10 × 10$^6$ CD4$^+$ T cells into CD8-depleted Hu-PBL-NOD-scid mice on the same schedule was unable to prevent the development of EBV-related LPD (7 of 9, 78%; Fig. 4). The depletion of donor NK cells by injection of anti-human CD16 mAb 1–3 days after PBMC engraftment had no effect on the proportion of mice that developed EBV-related LPD by 16 wk (6 of 17, 35%; Fig. 4).

Adaptive transfer of EBV-specific or allo-specific CTL into Hu-PBL-C.B-17-scid mice delays development of EBV-related lymphoproliferative disorders

We next determined whether EBV-specific CD8$^+$ T cells could delay or prevent EBV-related LPD in Hu-PBL-C.B-17-scid mice that develop this disorder at high frequency. To address this, we engrafted C.B-17-scid mice with 50–60 × 10$^6$ human PBMC from two different EBV-seropositive donors. Engraftment of these cell numbers is known to lead to the development of EBV-related LPD in the majority of scid mice (4, 34). One to 3 days after PBMC injection and twice weekly thereafter, 100–200 lytic units of CTL specific for autologous EBV$^+$ B-LCLs or allogeneic B-LCLs were injected i.p.

The cumulative survival of Hu-PBL-C.B-17-scid mice receiving either EBV-specific CTL (MST, 81 days; range, 49–95 days; $n = 13$) or allo-specific CTL (MST, 83 days; range, 62–89 days; $n = 9$) was significantly increased compared with that of untreated mice

<table>
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<tr>
<th>Recipient Strain</th>
<th>Ab Treatment</th>
<th>Human CD45$^+$ Cells (%)</th>
<th>Human CD45$^+$ Cells (×10$^6$)</th>
<th>Human CD4$^+$ Cells (%)</th>
<th>Human CD4$^+$ Cells (×10$^6$)</th>
<th>Human CD8$^+$ Cells (%)</th>
<th>Human CD8$^+$ Cells (×10$^6$)</th>
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<tr>
<td>NOD-scid</td>
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<td>55 ± 17</td>
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<td>51 ± 14</td>
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<td>48 ± 57</td>
<td>38 ± 20</td>
<td>40 ± 54*</td>
<td>&lt;1**</td>
<td>2 ± 3**</td>
</tr>
</tbody>
</table>

* The percentage and number of human CD45$^+$, CD4$^+$, and CD8$^+$ cells detected in the spleen of Hu-PBL-NOD-scid and Hu-PBL-NOD-scid-B2mnull mice 4 wk after injection of 20 × 10$^6$ human PBMC. Mice were untreated or treated with anti-CD154 mAb, anti-CD4 mAb, or anti-CD8 mAb as described in Materials and Methods. Values represent the mean ± SD of data derived from three to four individual experiments using different donors (9–20 mice in each experimental group).

* $p < 0.05$ as compared to untreated Hu-PBL-NOD-scid mice; **, $p < 0.05$ as compared to untreated Hu-PBL-NOD-scid and untreated Hu-PBL-NOD-scid-B2mnull mice.
MST, 62 days; range, 45–89 days; n = 23; p = 0.006 and p = 0.0008, respectively). Both types of CTL immunotherapy led to a delay in tumor outgrowth, but did not prevent tumors from developing. There was no statistical difference in the survival of recipients of EBV-specific and allo-specific CTLs (p = 0.26). Surviving mice were sacrificed on day 89 (untreated and allo-specific CTL-treated) or on day 95 (EBV-specific CTL-treated) and examined visually for evidence of EBV-related LPD. One untreated mouse (4.3%), three mice treated with EBV-specific CTL (23.1%), and two mice treated with alloreactive CTL (22.2%) were free of tumors at the end of the experiment, but these values were not statistically significant (p = 0.19, by χ² analysis).

**Discussion**

In the present report we have shown that the increased level of engraftment of human CD4⁺ and CD8⁺ cells in NOD-scid-B2mnull mice compared with that in NOD-scid mice (20) is due predominately to differences in levels of host innate immunity. Increased engraftment of human cells was not due to their differential stimulation by host MHC class I per se. The data further demonstrate that despite the increased numbers of human CD4⁺ cells in Hu-PBL-NOD-scid-B2mnull mice, the CD4⁺ cells still display phenotypic and functional characteristics of anergic cells. However, the data also demonstrate that human CD8⁺ cells have potent regulatory activity and control outgrowth of EBV lymphoblastoid cells in the PBMC-engrafted scid mice. Surprisingly, the specificity of human CD8⁺ CTL for EBV was not required to delay the development of EBV-related LPD in C.B-17-scid mice, but it is unknown whether EBV-specific or nonspecific CD8⁺ CTL are required to prevent the development of EBV-related LPD in Hu-PBL-NOD-scid mice.

Our previous studies have demonstrated that host strain characteristics influence the engraftment of human PBMC (17, 20). NOD-scid mice lack hemolytic complement, have defects in myeloid cells, and are relatively deficient in NK cell activity (19). NOD-scid-B2mnull mice retain all these innate immune deficiencies, but in addition are severely deficient in NK cell activity and lack murine MHC class I expression. NOD-scid-B2mnull mice support heightened levels of human T cell engraftment, and in contrast to that observed in Hu-PBL-NOD-scid mice, there was a normal ratio of CD4:CD8 human cells in Hu-PBL-NOD-scid-B2mnull mice (20). The basis of these differences in human cell engraftment could result from differences in host NK cell activity or from the absence of host MHC class I (20, 35, 36).

To dissect the mechanisms that drive human T cell engraftment in NOD-scid and NOD-scid-B2mnull mouse recipients, we evaluated the proliferation of human cells in the first week of engraftment in NOD-scid-B2mnull mice compared with that in NOD-scid mice. No differences in proliferation were observed in vitro in primary cultures following stimulation of fresh human PBMC with irradiated splenocytes from each strain. These results suggest that a deficiency in expression of MHC class I does not alter the direct

**FIGURE 3.** EBV-related lymphoproliferative disorders develop in CD8-depleted Hu-PBL-NOD-scid mice. NOD-scid mice were injected i.p. with 20 × 10⁶ human PBMC and given anti-CD8 mAb. Four weeks after cell engraftment tissues were examined histologically. Shown is a lymph node from a representative Hu-PBL-NOD-scid mouse treated with anti-CD8 mAb. A. Hematoxylin and eosin staining shows a diffuse proliferation of large lymphoid cells. B. In situ hybridization for the detection of EBER. The brown nuclear staining indicates an EBER-positive cell. C. Immunoperoxidase detection of CD3 shows scattered positive cells. D. Immunoperoxidase detection of CD20 shows numerous positive cells. Magnification in all panels, ×150.
proliferative stimulus of mouse splenocytes to human lymphocytes. Moreover, additional studies demonstrated that increased human T cell proliferation and a normal CD4:CD8 ratio could be obtained in NOD-scid mice following injection of TM-β1 anti-mouse CD122 mAb. IL-2R β-chain (CD122) is expressed on mouse NK cells, granulocytes, and, to a lesser extent, many other cell types (37, 38). Administration of TM-β1 mAb to Hu-PBL-C.B-17-scid mice has been shown to increase human cell engraftment (36). Our present data show that human cell proliferation is increased in NOD-scid mice treated with anti-mouse CD122 mAb, and that this treatment leads to a CD4:CD8 ratio similar to that observed in NOD-scid-B2mnull mice. Our interpretation of these results is that the high engraftment of CD4+ cells and the normal CD4:CD8 ratio observed in Hu-PBL-NOD-scid-B2mnull mice are the result of their almost total absence of NK activity.

In Hu-PBL-NOD-scid mice, the progression of human cells from naive to activated to an anergic phenotype proceeds concurrently in the peritoneal cavity and the spleen. These data are consistent with previous reports of low levels of activation 1 wk after engraftment (39) and expression of an anergic phenotype of human cells in the spleen of mice by 4–6 wk after engraftment (2). These observations suggest that the activation status of human cells does not determine whether they will localize in the spleen or peritoneal cavity in the scid host. By 4 wk, the proliferation of human cells in the spleen of NOD-scid mice is low and correlates kinetically with their loss of CD25 expression and acquisition of an anergic phenotype. Although overall levels of human cell engraftment and human CD4+ T cell engraftment were increased in NOD-scid mice, the human cells still displayed functional and phenotypic defects in activation. These phenotypic characteristics are reported to be associated with an anergic phenotype (2).

A new observation reported here is the regulation of human cell engraftment by human CD4+ and CD8+ cells. No detectable engraftment is observed in the absence of human CD4+ cells. These results demonstrate that there is a requirement for CD4-derived helper factors, perhaps IL-2, for the expansion of CD8+ cells in vivo. This help is probably induced through expression of CD154 and its interaction with CD40 on APC. In other studies we have observed that expansion of TCR transgenic alloreactive CD8+ cells in vivo is absolutely dependent on CD4 helper factors that are independent of CD40-CD154 interaction (40). These earlier results are similar to those obtained in the present experiments. The expression of CD25 on activated human cells at 1 wk, but not at 4 wk, after engraftment also supports this possibility, as does restoration of proliferation in vitro following addition of recombinant human IL-2.

We also document that the regulation of CD4+ cell expansion by CD8+ cells is a potentially novel model system with which to study CD8 regulatory activity in vivo. The mechanism by which this regulation occurs is unknown, but in other regulatory systems CD95- or TNF-mediated apoptosis, TGF-β, and T cell-NK cell interactions have all been implicated as mediators of suppression (41). In preliminary experiments we have obtained evidence that suppression of CD4+ cell engraftment may be mediated in part by elimination of activated CD4+ cells via CD95-CD95L (Fas-Fas ligand) cytotoxicity. Both CD4+ and CD8+ cells at 1 wk of engraftment express high levels of Fas, and preliminary in vitro experiments suggest that activated human CD4+ cells are more susceptible than activated human CD8+ cells to CD95L-mediated apoptosis (E. J. Wagar, unpublished observations).

The possible role of CD8+ cells as suppressor cells is consistent with several previous reports that have provided data on the in vivo function of CD8+ cells. These earlier studies have generated suggestive data that CD8+ cells (42–44) or anergic T cells can act as suppressor T cells (41, 45, 46). The mechanism of suppression has not been identified in these published studies.

Of additional interest is our documentation that Hu-PBL-NOD-scid mice are relatively resistant to the development of EBV-related LPD. Inhibition of EBV-related LPD in Hu-PBL-NOD-scid mice may be mediated by the increased levels of engraftment of human CD8+ cells (47–49). This interpretation is based on previous data obtained in humans (50–53) and in scid mice bearing EBV-related LPDs (54). In humans, transfer of autologous EBV-specific CTL prevents or delays the development of EBV-related LPD following bone marrow (50–52) or solid organ transplantation (53). In mice bearing B-LCL tumors, autologous EBV-specific CTLs can induce the regression of coinjected or established B-LCL tumors in the host (54). Our data suggest that human CD8+ cells in NOD-scid mice remain functional and mediate the suppression of EBV-related LPD outgrowth. This inference was confirmed by data obtained in CD8-depleted Hu-PBL-NOD-scid mice and in CD8-depleted mice that were reconstituted with human CD8+ cells. Published data suggest that human EBV-specific CTLs can induce regression against established EBV-related LPD in scid mice (54). Our current data suggest further that human CD8+ cells also provide surveillance activity to prevent the development of EBV-related LPD in Hu-PBL-C.B-17-scid mice. This observation is consistent with the data in humans demonstrating that IL-2-activated CD8+ T cell immunotherapy can prevent post-transplant LPD (55). The requirement for EBV-specific CTL vs nonspecifically activated CD8+ T cells in surveillance is under investigation. Our studies also ruled out a major role for human NK cell control of EBV-related LPD.

We also observed that CD8+ cells regulated human CD4+ cell outgrowth. This may be due to a direct suppressive effect, as described above, or indirectly by prevention of EBV-related LPD outgrowth. The expanding EBV+ cells would provide high levels of human MHC class II and multiple costimulatory factors that are
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normally absent in Hu-PBL-NOD-scid mice. This, in turn, could stimulate human CD4+ cell proliferation. Finally, human CD8+ cells appeared to modulate the levels of circulating human Ig. This effect could be directly on Ig-producing B lymphocytes or a secondary effect due to regulating the expansion of EBV-infected cells. These data document the important role of CD8-mediated inhibition of B cell activation, and the complex balance between CD4 help and CD8 suppression on B cell function and lymphoproliferation in the human immune system.

Surprisingly, the lack of MHC class I expression by NOD-scid-B2m−/− host cells did not appear to affect the activation or proliferation of engrafted human CD8+ cells. These data suggest that direct Ag presentation via MHC class I by mouse APC to human CD8+ cells is probably not the mechanism by which human CD8+ cells are activated. This conclusion is consistent with reports by others that indirect Ag presentation is the primary mechanism responsible for the proliferation of human T cells in a human-mouse xenogeneic mixed lymphocyte culture (56) and in the generation of human CTLs (57). Recently, however, the role of T-T Ag presentation has been highlighted (58, 59). Because the human T cells engrafted in scid mice are activated and express MHC class II molecules, T-T cell Ag presentation may also be important in their activation, proliferation, and development of an anergic phenotype. Alternatively, the costimulation and activation of human T cells may be dependent on murine host APC, as our previous data have shown that anti-mouse CD40 mAb also prevents engraftment of human T cells (20). These data, taken together, suggest that activation of human T cells may be dependent on indirect or T-T Ag presentation but independent of coactivation between human CD154 and human CD40.

It should be cautioned, however, that most human lymphocytes in scid mice are chronically activated, an observation noted in our present studies and in previously reported studies (4, 60). This suggests that CD8+ cell regulation in our model system may be mediated by memory/activated secondary responses rather than primary responses of naive T cells. Overall, based on the present data, we propose that the Hu-PBL-NOD-scid model may be suitable for the in vivo analysis of human CD4+ and CD8+ T cells and of immune regulation of EBV-related LP development.

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References


