Expanded CD34$^+$ Human Umbilical Cord Blood Cells Generate Multiple Lymphohematopoietic Lineages in NOD-*scid IL2rγ^{null}* Mice

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Umbilical cord blood (UCB) is increasingly being used for human hematopoietic stem cell (HSC) transplantation in children but often requires pooling multiple cords to obtain sufficient numbers for transplantation in adults. To overcome this limitation, we have used an *ex vivo* two-week culture system to expand the number of hematopoietic CD34$^+$ cells in cord blood. To assess the *in vivo* function of these expanded CD34$^+$ cells, cultured human UCB containing 1 × 10^6 CD34$^+$ cells were transplanted into conditioned NOD-*scid IL2rγ^{null}* mice. The expanded CD34$^+$ cells displayed short- and long-term repopulating cell activity. The cultured human cells differentiated into myeloid, B-lymphoid, and erythroid lineages, but not T lymphocytes. Administration of human recombinant TNFα to recipient mice immediately prior to transplantation promoted human thymocyte and T-cell development. These T cells proliferated vigorously in response to TCR cross-linking by anti-CD3 antibody. Engrafted TNFα-treated mice generated antibodies in response to T-dependent and T-independent immunization, which was enhanced when mice were co-treated with the B cell cytokine BLyS. *Ex vivo* expanded CD34$^+$ human UCB cells have the capacity to generate multiple hematopoietic lineages and a functional human immune system upon transplantation into TNFα-treated NOD-*scid IL2rγ^{null}* mice. Exp Biol Med 233:997–1012, 2008

Key words: NOD-*scid IL2rγ^{null}* mice; SCID; umbilical cord blood; TNFα; *ex vivo* expansion; human; immune response

Introduction

Allogeneic hematopoietic stem cell (HSC) transplantation following myeloablative therapy is curative for many malignant, genetic, and autoimmune disorders (1). Sources of stem cells for clinical transplantation include bone marrow, cytokine-mobilized peripheral blood, and umbilical cord blood (UCB) (2–5). Human UCB is increasingly used as a clinical source of HSC due to the advantages including immediate availability, high proliferative capacity, and the low incidence of severe GVHD in recipients (4, 6–13). However, successful human HSC transplantation is dependent on the absolute number of CD34$^+$ cells transplanted (14–19). In the case of UCB, this often requires the pooling of multiple cords to obtain the optimal CD34$^+$ cell dose (17, 18, 20). Concerns related to the use of pooled cords include the possibility of graft-versus-graft as well as the unpredictable reconstitution of individual cords (17, 19, 20).

Many laboratories are culturing UCB to increase the CD34$^+$ cell yield from a single cord (21–25). Long-term cultures have been successful in expanding CD34$^+$ cell numbers, but maintaining HSC activity has been difficult

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Table 1. $CD34^+$ Cell Expansion in Two-Week Culture[a]

Cell phenotype	Cell input	Cell yield after two week culture	Fold increase in cell number
CD45 cells	$3.8 \pm 0.9 \times 10^6$	$2.6 \pm 1.1 \times 10^8$	68.4-fold
CD34 cells	$7.1 \pm 1.3 \times 10^5$	$2.6 \pm 0.9 \times 10^7$	36.6-fold

[a] Human UCB was cultured as described in Materials and Methods. The number of each cell population after RBC and lineage depletion that was put into culture or recovered after two weeks of culture is shown. Shown are the numbers of $CD45^+$ cells (total human leukocytes) and total human $CD45^+CD34^+$ cells (total human $CD34^+$ cells). Results represent the mean ± SEM of at least 30 separate cultures.

(26–30). Investigators rely on immunodeficient mice, particularly NOD-*scid* mice, to define the *in vivo* function of the cultured HSC. However, transplantation of cultured human HSC into NOD-*scid* mice has yielded only low engraftment, limited multilineage hematopoietic development, and few if any HSC-derived T lymphocytes (31–33).

Recently, new immunodeficient murine mouse models that lack a functional common IL-2-receptor gamma chain (*IL2rγ*^{null}) have been found to be superior in their support of human HSC engraftment (34). These animals lack mature lymphocytes and NK cells, express other impairments in innate immunity, and are long-lived, permitting long-term studies of engraftment with human HSCs (34).

In the present study, we used the NOD-*scid IL2rγ*^{null} mouse model to investigate the *in vivo* potential of human UCB stem cells following expansion of $CD34^+$ cells in a two-week culture system. Cultured UCB engrafted and developed into myeloid, erythroid, and B-lymphoid lineages, and when the recipients were pre-treated with TNFα, functional T lymphoid cells were generated. Engrafted mice produced antibody in response to immunization with T-dependent and T-independent antigens, which was enhanced by administration of the human B Lymphocyte Stimulator (BLyS) cytokine (also termed BAFF or TNFSF13B).

Materials and Methods

Mice. NOD.CB17-*Prkdc*^{scid} (abbreviated as NOD-*scid*) and NOD.Cg-*Prkdc*^{scid} *IL2rg*^{tmWjl}/Sz (abbreviated as NOD-*scid IL2rγ*^{null}) mice have been described (35, 36). Male and female 6- to 12-week-old mice of each strain were used in these studies. They were specific pathogen free, housed in microisolator cages, and given autoclaved food and on alternate weeks given autoclaved acidified water or sulfamethoxazole-trimethoprim–medicated water (Goldline Laboratories, Ft. Lauderdale, FL). All animal use was in accordance with the guidelines of the Animal Care and Use Committees of the University of Massachusetts Medical School and The Jackson Laboratory.

Antibodies. Anti-human α/β TCR was obtained from Becton Dickinson (San Jose, CA). Anti-human BDCA2 was obtained from Miltenyi (Auburn, CA). All other antibodies and isotype controls were obtained from BD Pharmingen (San Diego, CA). Antibodies were conjugated with FITC, PE, PerCP, APC, Alexa 405, Pacific Blue, or Alexa700.

Cell Preparation. Single-cell suspensions of bone marrow, spleen, and thymus were prepared from engrafted mice. Whole blood was collected in PBS containing EDTA. RBC in marrow and spleen were removed by lysis with a hypotonic solution. GlyA-stained bone marrow cells were not subjected to red blood cell lysis. Cells counts were performed using a Coulter Counter (Beckman, Miami, FL).

Flow Cytometry. Single cell suspensions or whole blood were preincubated with rat anti-mouse FcR11b (2.4G2) to block Fc binding and then incubated with appropriate dilutions of specific antibodies. In all wells, PerCP-conjugated anti-mouse CD45 and APC-conjugated anti-human CD45 were included. Labeled cells were fixed in 1% paraformaldehyde for analysis. Whole blood was stained according to manufacturer's instructions (BD Biosciences, San Jose, CA). At least 50,000 events were acquired on either BD Biosciences LSRII or FACSCalibur instruments (BD Biosciences). All cells that were murine $CD45^-$ and human $CD45^+$ were analyzed for the presence of multilineage differentiation. Bone marrow cells that were negative for murine CD45 were also analyzed for the presence of human RBC and their progenitors. Data acquisition and analysis was performed with BD FACSDiva (BD Pharmingen) and FlowJo (Tree Star, Inc., Ashland, OR) software.

***Ex Vivo* Culture of $CD34^+$ Human UCB Stem Cells.** Cultured UCB stem cells were prepared by Viacell, Inc. (Cambridge, MA). Briefly, red blood cells were removed using a double-density Percoll Gradient (1.05/1.077 g/ml). The recovered UCB cells were lineage-depleted using Stem Cell Technologies (Vancouver, British Columbia, Canada) SCT 9 antibody cocktail (CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b, GlyA). The lineage-depleted cells were cultured for seven days in Stemspan (Stem Cell Technologies) supplemented with defined lipid cocktail (Gibco, Carlsbad, CA), 50 μg/mL gentamycin (Abbott Laboratories, Chicago, IL), 100 ng/mL SCF (Amgen, Thousand Oaks, CA), 100 ng/ml Flt3-L (Amgen), and 100 ng/ml Tpo (R&D Systems, Minneapolis, MN). At day seven of culture, non-adherent cells were recovered, lineage depletion was repeated, and cells were cultured for an additional seven days. After two weeks in culture, the percentage and number of cell subsets was determined by flow cytometry.

Engraftment of Mice with Cultured $CD34^+$ Human UCB Stem Cells. Adult NOD-*scid IL2rγ*^{null} and NOD-*scid* mice were irradiated with 2.4 and 3.25 Gy,

Table 2. Phenotype of Human UCB Prior to and After a Two-Week Culture[a]

Phenotype	Cell population	Input cell population (mean ± SEM)	Cultured cell population (mean ± SEM)
CD34	Hematopoietic progenitor	32.3 ± 3.9	20.1 ± 1.9
CD34/CD33/CD13	Myelomonocytic stem cell	0.1 ± 0.1	12.9 ± 1.2
CD33/CD13	General myeloid precursor	9.0 ± 2.5	53.6 ± 2.7
CD34/CD7	Lymphoid/NK progenitor	0.6 ± 0.4	1.2 ± 0.3
CD41/CD61	Megakaryocyte	13.5 ± 1.8	23.0 ± 1.8
CD34/CD41/CD61	Megakaryocyte progenitor	2.5 ± 1.0	2.7 ± 0.5
CD3	T lymphoid lineage	0.3 ± 0.1	0.8 ± 0.2
CD14	Monocyte	0.3 ± 0.11	4.2 ± 1.0
CD19	B lymphoid lineage	0.9 ± 0.3	0.4 ± 0.1
GlyA	Immature and mature erythrocyte	3.7 ± 2.1	1.6 ± 0.7

[a] Human UCB was cultured as described in Materials and Methods. The relative percentage of each cell population after RBC and lineage depletion that was put into culture or recovered after two weeks of culture is shown. Results represent the mean ± SEM of at least 20 separate cultures. All values represent the percent mean ± SEM of human $CD45^+$ cells with the exception of GlyA, which were derived from the $CD45^-$ population.

respectively, from a ^{137}Cs source (Gammacell 40, Atomic Energy of Canada, Ottawa, Canada) using previously optimized irradiation doses (36, 37). Mice were injected intravenously with cultured cells containing 1×10^6 $CD34^+$ cells.

TNF α Treatment. Recombinant human TNFα (R&D Systems) was reconstituted in PBS (10.0 μg/ml) containing 0.1% BSA. A single injection of 50 μl of TNFα (0.5 μg/recipient) was given i.p. immediately after irradiation and 4 hours before injection of human UCB cells.

Immunization. NOD-*scid IL2r*γ^{null} mice engrafted with cultured $CD34^+$ cells 12 weeks earlier were immunized s.c. with 20 μl/mouse containing 1 μg each of 23 pneumococcal serotypes (Pneumovax 23; Merck, Whitehouse Station, NJ). Serum was recovered 14 days after primary immunization. Mice were then injected i.p. with BLyS (10 μg) or PBS for 4 days, and reboosted with Pneumovax 28 days after primary immunization. BLyS was made from recombinant material from a cell line provided by Dr. Randoph Noelle (Dartmouth Medical, Lebanon, NH). Serum was recovered 14 days after boosting for analysis. The same mice were then immunized s.c. 8 days later with Tetanus Toxoid Adsorbed USP (0.15 μl; Aventis Pasteur Inc., Swiftwater, PA), and serum was obtained 14 days later. Three mice that had previously been given BLyS (see above) were again treated with BLyS for 4 days and reboosted with Tetanus Toxoid. Serum was recovered 14 days later for analysis.

Table 3. Lineage Marker Expression on Engrafted Human $CD45^+$ Cells in the Bone Marrow of NOD-*scid IL2r*γ^{null} Mice Three Weeks After Transplantation of Cultured Human UCB[a]

Lineage	Phenotype	Percent (mean ± SEM, $n = 11$)
	CD45	2.3 ± 0.4
Phenotype of $CD45^+$ cells		
	CD34	17.1 ± 1.4
	CD33	87.3 ± 4.4
	CD38	94.1 ± 1.2
	CD33CD64	23.5 ± 2.6
	CD20	11.1 ± 1.7
	CD3	<0.05
	CD71	0.1 ± 0.1
	Gly A	0.1 ± 0.1

[a] Irradiated NOD-*scid IL2r*γ^{null} mice were transplanted with cultured human UCB containing 1×10^6 $CD34^+$ cells. Bone marrow was analyzed by flow cytometry for the presence of human myeloid and lymphoid lineages three weeks after transplantation. Shown is the percent of human $CD45^+$ bone marrow cells and lineage phenotype data are expressed as percent of human $CD45^+$ cells.

Human Ig ELISA. Levels of human IgG and IgM were measured in mouse sera using an ELISA assay. Briefly, ELISA plates were coated with the $F(ab')_2$ fragment of anti-human IgG or IgM (Jackson ImmunoResearch, West Grove, PA) overnight and blocked with 0.5% horse serum and 0.02% azide in PBS. Triplicate serial dilutions of sera were added, followed by biotinylated mouse anti-human IgL kappa or lambda (Ebiosience, San Diego, CA). Streptavidin alkaline phosphatase (Southern Biotech) was added, and the reaction visualized by addition of 1 mg/ml *p*-nitrophenyl phosphate (Sigma-Aldrich) in 1 M diethanolamine, 0.5 mM $MgCl_2$. Color development was terminated after 30 min with 3M NaOH, and plates were read at 405 nm using an Emax ELISA plate reader (Molecular Devices, Sunnyvale, CA). The levels of human IgG and IgM in the sera were calculated using a standard curve (Jackson ImmunoResearch). Limits of detection were 3 ng/ml for IgM and 2 ng/ml for IgG.

Streptococcus Pneumonia ELISA. Antibodies specific for *Streptococcus pneumoniae* capsular polysaccharide serotypes 4 or 14 were measured in mouse sera as described (38). The levels of antibody in the sera were calculated using a standard (US reference human pneumococcal antiserum; FDA, Rockville, MD).

Tetanus ELISA. Quantification of anti-tetanus antibody used a commercial ELISA kit (SCIMEDX Corpo-

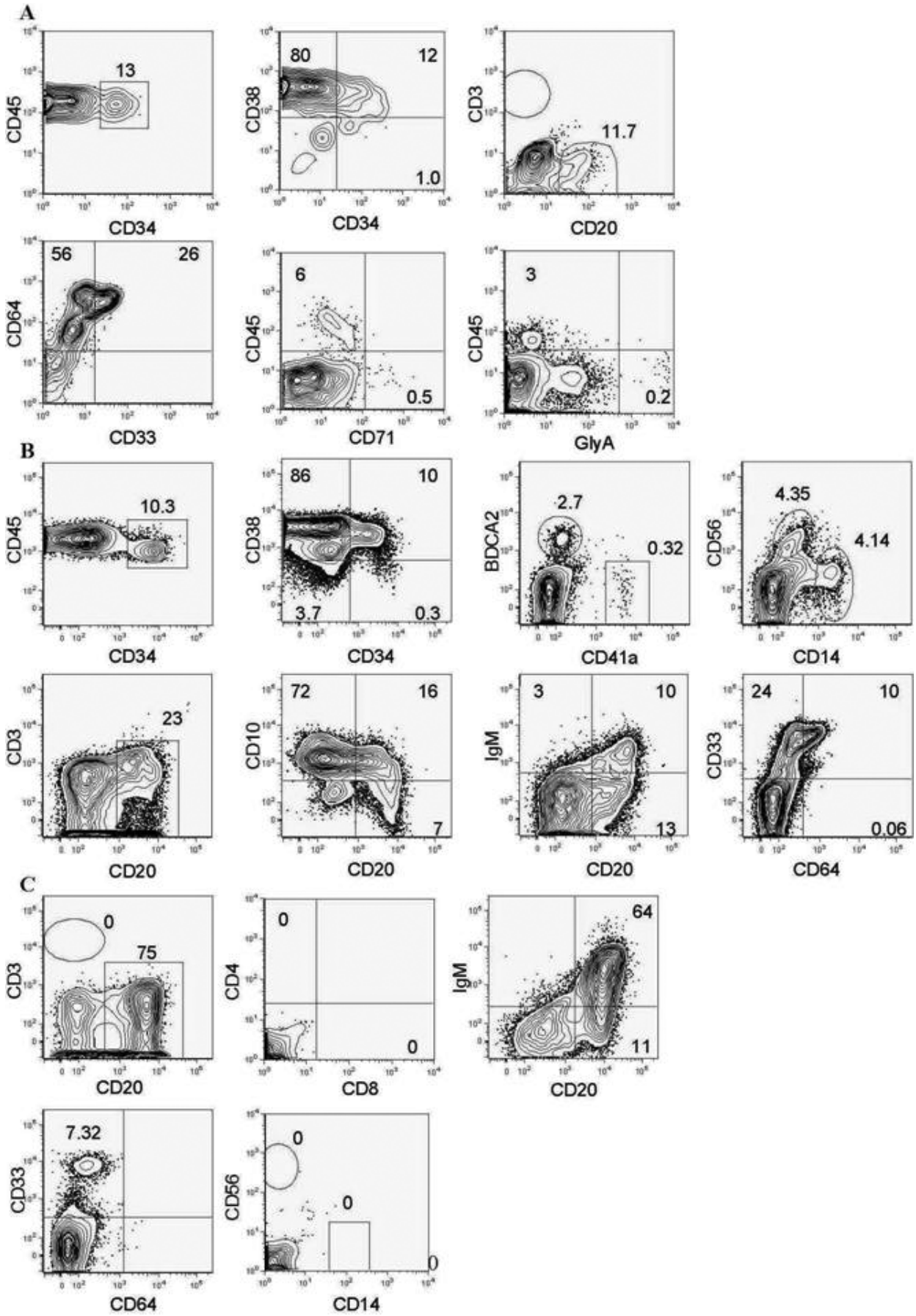

Table 4. Lineage Marker Expression on Engrafted Human CD45$^+$ Cells in Non-TNFα-Treated and TNFα-Treated NOD-*scid IL2rγ*^{null} Mice Twelve Weeks After Transplantation of Cultured Human UCB[a]

		Bone marrow		Spleen	
Lineage	Phenotype (% of CD45$^+$ cells)	No TNFα (mean ± SEM, $n = 26$)	TNFα (mean ± SEM, $n = 19$)	No TNFα (mean ± SEM, $n = 12$)	TNFα (mean ± SEM, $n = 11$)
Stem/progenitor	CD34	10.3 ± 1.0	9.7 ± 1.6	N.D.	N.D.
	CD38/CD34	9.9 ± 1.1	8.7 ± 1.3	N.D.	N.D.
Myeloid	CD33	30.0 ± 3.6	27.5 ± 3.6	6.0 ± 1.7	6.4 ± 2.1
	CD33/CD64	12.1 ± 2.1	7.2 ± 1.1	<0.05	<0.05
	CD14	5.1 ± 0.5	6.0 ± 0.8	<0.05	0.1 ± 0.1
	CD38	93.0 ± 2.1	95.1 ± 1.4	N.D.	N.D.
	CD41a	0.3 ± 0.1	0.2 ± 0.1	N.D.	N.D.
	BDCA2	3.3 ± 0.3	3.9 ± 0.7	N.D.	N.D.
	CD71	0.4 ± 0.3	2.0 ± 0.8	N.D.	N.D.
Lymphoid	CD20	26.5 ± 1.7	21.2 ± 1.9	81.9 ± 2.0	70.2 ± 3.8
	CD20/IgM	15.4 ± 1.1	11.6 ± 1.3	66.0 ± 1.8	49.3 ± 4.1
	CD20/CD10	14.2 ± 1.7	11.4 ± 2.4	N.D.	N.D.
	CD3	<0.05	<0.05	0.1 ± 0.1	10.2 ± 6.2
	CD56	2.4 ± 0.5	3.6 ± 1.0	<0.05	0.1 ± 0.1
Erythroid	GlyA	0.5 ± 0.1	1.7 ± 0.4	N.D.	N.D.

[a] Irradiated non-TNFα-treated and TNFα-treated NOD-*scid IL2rγ*^{null} mice were transplanted with cultured human UCB containing 1×10^6 CD34$^+$ cells. Bone marrow and spleen were analyzed by flow cytometry for the presence of human myeloid and lymphoid lineages twelve weeks after transplantation. Data are expressed as percent of human CD45$^+$ cells; $n = 9$–12/group; N.D., not determined.

ration, Denville, NJ). The ELISA was performed according to the manufacturer's instructions, and developed for visualization using sheep anti-human horseradish peroxidase conjugate. The optical density at 450/620 was measured on a MDS Analytical Technologies Emax Precision Microplate Reader (Toronto, Canada). All tetanus antibody titers were calculated based on standards included with the ELISA kit and are reported as milli-International Units per milliliter (mIU/ml).

Proliferative Response Following Stimulation with Anti-CD3 Antibody. Splenocytes from TNFα-treated NOD-*scid IL2rγ*^{null} mice engrafted with cultured UCB were plated in anti-CD3 mAb coated wells of a flat bottom 96-well plate (Costar, Cambridge, MA) in triplicate at a concentration of 2.5×10^5 cells/well in 200 μl RPMI supplemented with 10% FBS. PBMC from a normal donor was used as a positive control. Cells were stimulated with plate bound anti-CD3 mAb and soluble anti-CD28/CD49d FastImmune reagent (1 μg/ml, BD Pharmingen, San Diego, CA) for 64 hrs at 37°C in an atmosphere of 5% CO_2, and 1 μCi of [^{3}H]thymidine (Perkin Elmer, Waltham, MA) was added during the last 16 hrs of culture. [^{3}H]thymidine incorporation was measured using a 1450 Microbeta Trilux Instrument (Wallac, Gaithersburg, MD). The number of CD3$^+$ cells in each sample was determined and used to calculate the counts/CD3$^+$ T cell. Data are presented as mean cpm/1×10^5 human CD3$^+$ cells ± 1 SEM.

Histology. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5 μm. Antigen retrieval prior to immunohistochemical staining was performed by incubating the sections in 0.1 mol/l citrate buffer (pH 6.0) in an 800-W microwave oven for 15 min. The slides were stained on a Dako (Carpinteria, CA) automated immunostainer using the EnVision (Dako) staining procedure. The sections were incubated with the EnVision plus Dual Link reagent (a polymer conjugated with goat anti-rabbit Ig or goat anti-mouse Ig and horseradish peroxidase) for 30 min. The sections were reacted with diaminobenzidine and hydrogen peroxide for visualization and counterstained with hematoxylin. Negative controls induced species matched non-binding antibodies. A qualified pathologist (BAW) who was unaware of the treatment of the tissue donor evaluated all tissues.

Statistics. Results are expressed as mean ± one standard error. Values of specified groups were compared with the unpaired Student's t test (GraphPad Software, Version 4.0, San Diego, CA). Statistical significance was assumed for P values of <0.05.

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Figure 1. Lineage marker expression on engrafted human CD45$^+$ cells in NOD-*scid IL2rγ*^{null} mice. NOD-*scid IL2rγ*^{null} mice were transplanted with 1×10^6 cultured CD34$^+$ human UCB cells. Bone marrow was analyzed by flow cytometry for the presence of human myeloid and lymphoid lineages three (A) and twelve (B) weeks after transplantation and spleen was analyzed twelve weeks (C) after transplantation. Total human cells are shown as the percent positive human CD45$^+$ cells. Lineage markers are expressed as percent of human CD45$^+$ cells in the bone marrow. CD71 and GlyA populations are expressed as percent of murine CD45$^-$ cells. Shown are representative histograms.

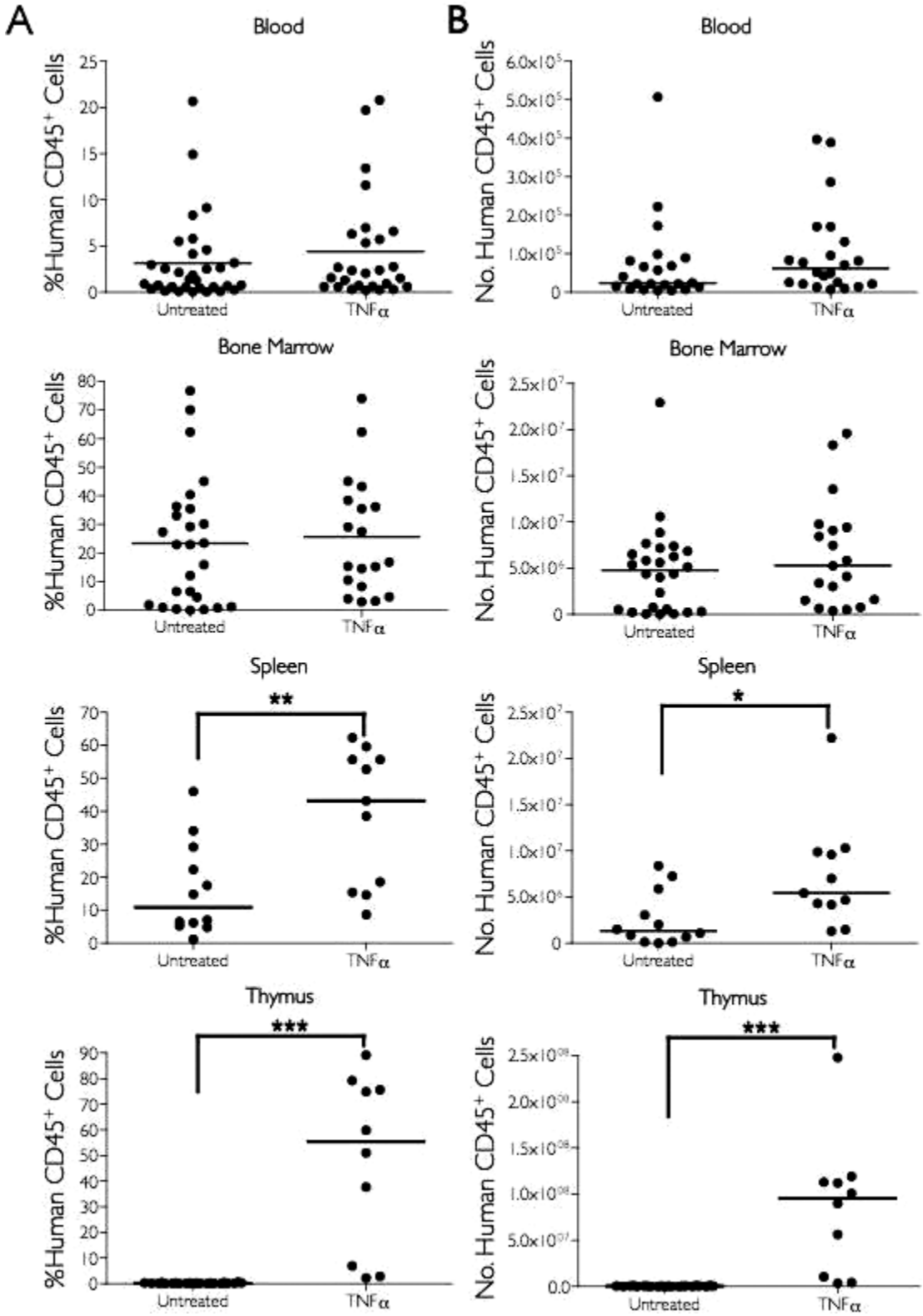

Figure 2. Engraftment of human CD45$^+$ cells in non-TNFα-treated and TNFα-treated NOD-*scid IL2rγ^{null}* mice 12 weeks after transplantation of cultured human UCB. Irradiated non-TNFα-treated and TNFα-treated NOD-*scid IL2rγ^{null}* mice were transplanted with 1×10^6 cultured CD34$^+$ human UCB cells. (A) Percent and (B) number of human CD45$^+$ cells in blood, bone marrow, spleen, and thymus twelve weeks after transplantation. $^*P < 0.025$, $^{**}P < 0.01$, $^{***}P < 0.001$.

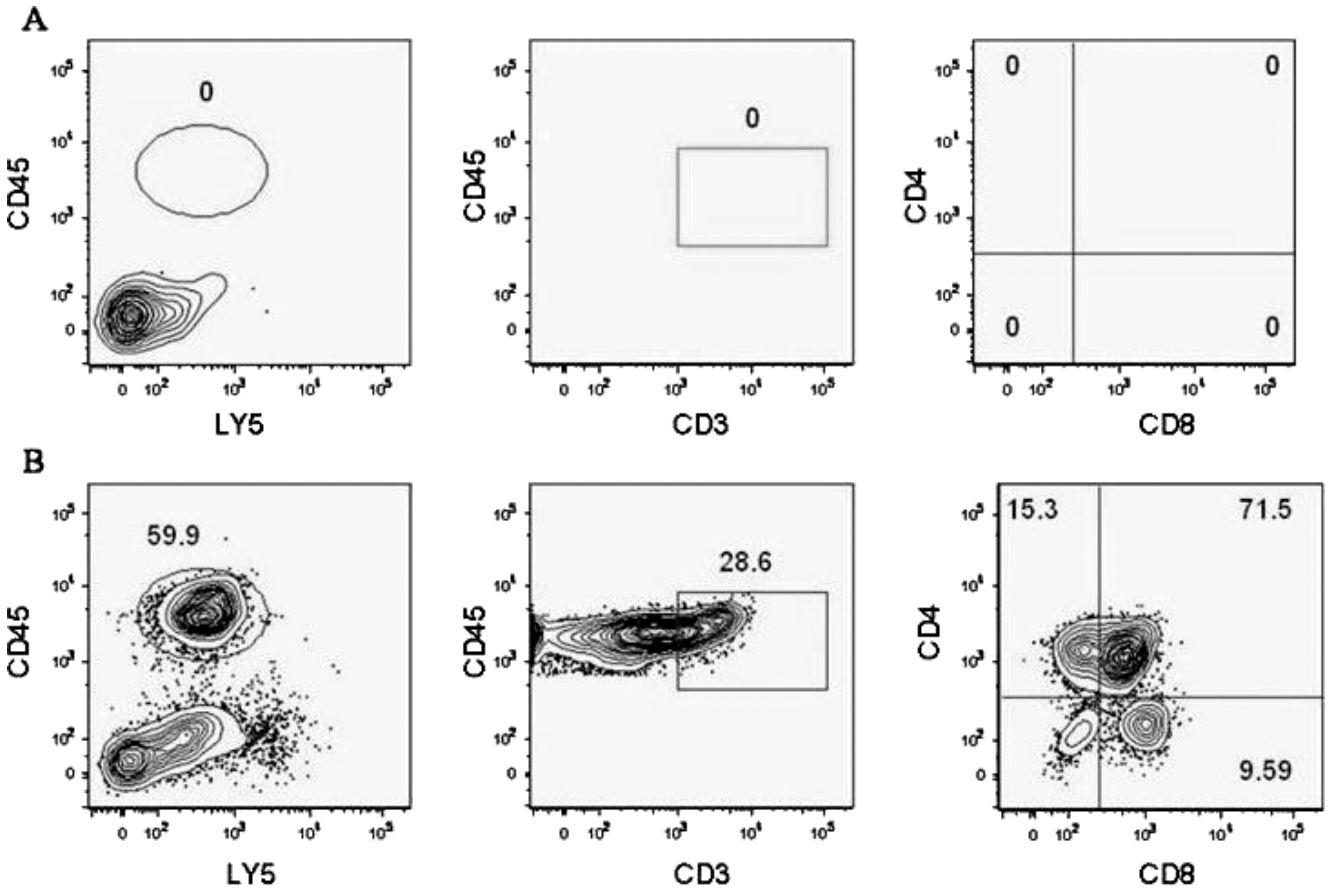

Figure 3. Phenotype of human CD45$^+$ cells in the thymus of non-TNFα-treated and TNFα-treated NOD-*scid IL2rγ*^{null} twelve weeks after transplantation of cultured human UCB. Irradiated non-TNFα-treated and TNFα-treated NOD-*scid IL2rγ*^{null} mice were transplanted with 1×10^6 cultured CD34$^+$ human UCB cells. Twelve weeks later, thymi were analyzed by flow cytometry. Representative flow cytometric profiles of human CD45$^+$ cell development in (A) non-TNFα-treated and (B) TNFα-treated NOD-*scid IL2rγ*^{null} mice. Human CD3 percentages are expressed as percent human CD45$^+$ cells. Human CD4 and CD8 percentages are expressed as percent human CD3$^+$ cells.

Results

***Ex Vivo* Expansion of CD34$^+$ Human UCB.** *Phenotype of Cells Recovered from a Two-Week Culture of Human UCB.* Human UCB, cord blood cells were cultured for two weeks in the presence of a cytokine cocktail, and the number and phenotype of the nonadherent cells was determined. The average expansion of human CD45$^+$ cells and CD34$^+$ cells was ~68-fold and ~37-fold, respectively (Table 1). The UCB population put into culture was devoid of mature lymphoid and myeloid populations and contained 32.3 ± 3.9% CD34$^+$ cells (Table 2). Although the percentage of CD34$^+$ cells in the population recovered from culture (20.1 ± 1.9%) was slightly lower than in the input population, the overall expansion led to an increase in the total number of CD34$^+$ cells recovered.

Phenotypically, many two-week cultured CD34$^+$ cells co-expressed CD33 and CD13, suggesting they were early stem/progenitor cells in the granulocyte and monocyte lineages (Table 2). Less than 1% of the cells recovered from culture were CD3$^+$ or CD19$^+$, less than 2% of the cells were GlyA$^+$, and less than 5% of recovered cells were CD14$^+$. Approximately 1% of the total cells were CD34$^+$CD7$^+$, suggesting a commitment to a T or NK cell lineage. Megakaryocytic lineage progenitors (CD34$^+$CD41$^+$CD61$^+$) comprised ~3% of the recovered cells (Table 2).

Engraftment of Cultured Human UCB in NOD-scid and NOD-scid IL2rγ^{null} *mice.* The NOD-*scid* mouse has been the "gold standard" for *in vivo* analyses of human HSC for over 10 years (36, 37, 39–43). We and others have recently described new immunodeficient mouse models based on the targeted mutation in the IL-2 receptor common γ chain (34). Therefore, we first compared the ability of cultured UCB to engraft in NOD-*scid* and NOD-*scid IL2rγ*^{null} mice. These stocks of mice were injected with cultured human UCB cells containing 1×10^6 CD34$^+$ cells and engraftment in the bone marrow was quantified twelve weeks later.

Low levels of human CD45$^+$cell engraftment were observed in the bone marrow of NOD-*scid* mice (0.2 ± 0.1%, $n = 8$). In contrast, robust levels of human CD45$^+$cells were generated in NOD-*scid IL2rγ*^{null} mice (24.3 ± 6.6%, $n = 5$, $P < 0.001$). Based on these data, all subsequent experiments were performed using NOD-*scid IL2rγ*^{null} mice as recipients.

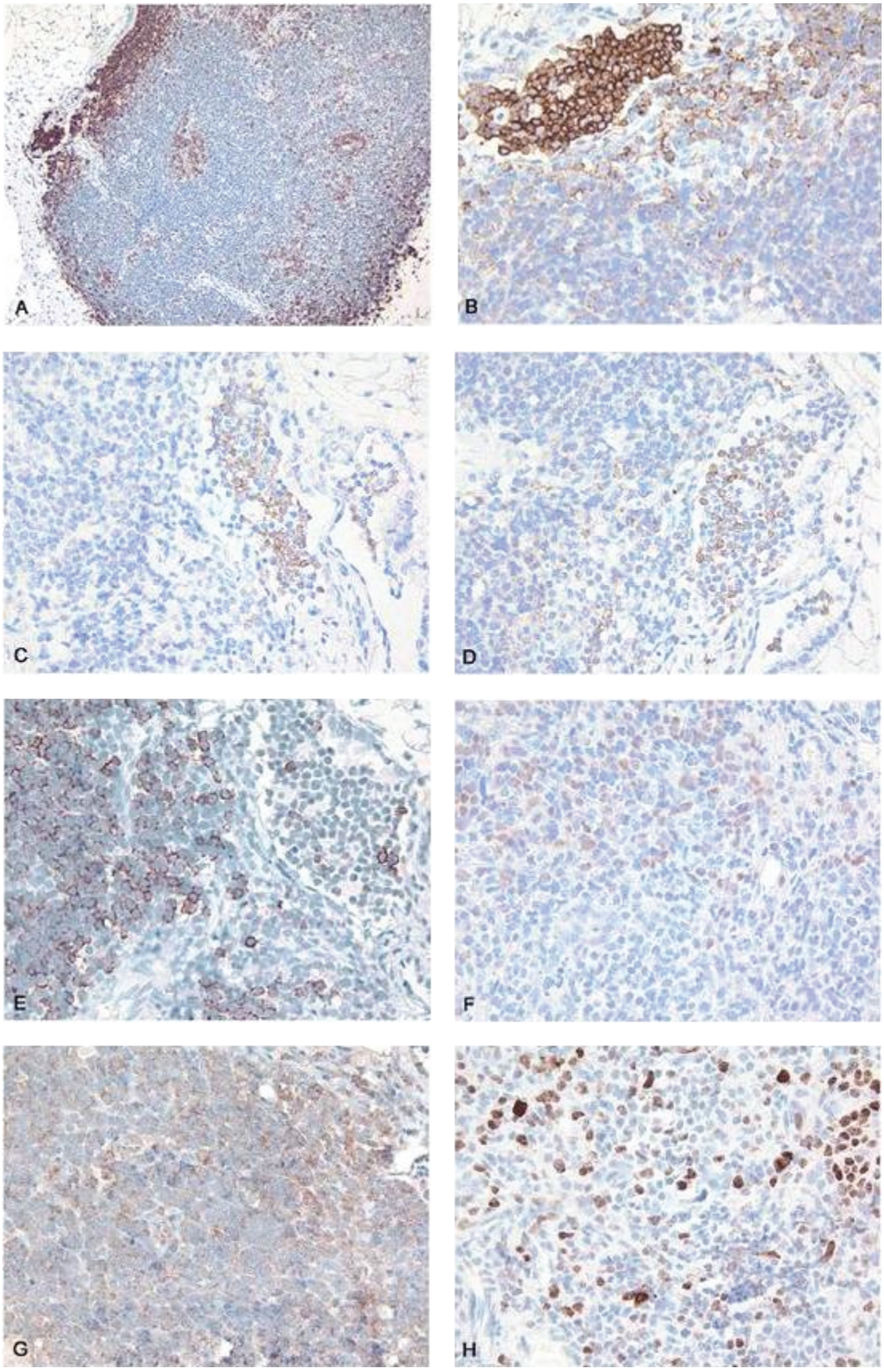

Figure 4. Immunohistochemistry of thymi of TNFα-treated NOD-*scid IL2rγ*^{null} mice twelve weeks after transplantation of cultured human UCB. Irradiated TNFα-treated NOD-*scid IL2rγ*^{null} mice were transplanted with 1×10^6 cultured CD34$^+$ human UCB cells. Twelve weeks later, thymi

Short- and Long-Term Stem Cell Repopulating Potential of Cultured Human UCB. Two major human stem/progenitor cell populations have been described using immunodeficient mice engrafted with human HSC: one is termed short-term repopulating cell (STRC), the other long-term repopulating cell (LTRC) (44, 45).

Short-Term Repopulating Cell Engraftment. Cultured human UCB containing 1×10^6 $CD34^+$ cells were injected into conditioned NOD-*scid IL2rγ*^{null} mice, and human cell engraftment in the bone marrow of the recipients was determined 3 weeks later.

Human $CD45^+$ cells comprised ~2% of total bone marrow cells (Table 3). To determine their lineage, we analyzed the subsets of human $CD45^+$ cells present in the marrow, specifically analyzing for the presence of cell populations that are known to comprise the short-term repopulation cell population in *scid* mice (44, 45). Of the $CD45^+$ cells, $CD34^+$ stem/progenitor cells were generated, including many committed to the myeloid ($CD33^+$ and/or $CD38^+$) lineage. $CD45^+$ cells expressing phenotypic characteristics of early granulocytes, monocytes, and macrophages ($CD33^+CD64^+$) were also observed. In the lymphoid lineage, $CD20^+$ B cells were generated, but no detectable $CD3^+$ T cells were observed (Table 3). Representative histograms are shown in Figure 1. These data demonstrate that cultured UCB display human STRC capacity when transplanted into NOD-*scid IL2rγ*^{null} mice.

Long-Term Repopulating Cell Engraftment. We next analyzed the subsets of human cells known to comprise the long-term repopulation cell population in *scid* mice (36). We determined if cultured UCB could generate long-term engraftment, multiple lineages of immature and mature cells, and hematopoietic stem cells in NOD-*scid IL2rγ*^{null} mice. Bone marrow and spleen were recovered 12 weeks after injection of cultured UCB and analyzed for the presence of human cells.

Bone Marrow. Human $CD45^+$ cells (23.3 ± 4.4%) expressing phenotypes associated with multilineage hematopoietic cell populations were readily detected in the bone marrow (Table 4). Myeloid progenitors expressing CD33 as well as more mature $CD64^+CD33^+$ myeloid cells were detected. $CD14^+$ monocytes, $CD41a^+$ megakaryocytes, and $BDCA2^+$ dendritic cells were also observed (Table 4). In the lymphoid lineage, $CD10^+CD20^+$ B cells were detected and some B cells coexpressed both CD20 and cell surface IgM. Human $CD56^+$ NK cells were also detected (Table 4). These data document that the cultured cells could generate both lymphoid and myeloid lineage populations.

Spleen. Human $CD45^+$ cells were also detected in the spleen twelve weeks after injection (16.2 ± 4.1%). These $CD45^+$ cells were comprised of multiple hematopoietic lineages. $CD20^+IgM^+$ B cells and $CD33^+$ myeloid progenitor cells were observed. However, no human $CD3^+$ T cells were detected (Table 4). Bone marrow recovered from a cohort of these mice was adoptively transferred into secondary NOD-*scid IL2rγ*^{null} mice. Twelve weeks later, human $CD45^+$ cells were detected in 6 of 8 of the secondary recipients, suggesting the presence of HSC in the primary hosts. These data demonstrate that cultured UCB display human LTRC activity but fail to generate mature T cells when transplanted into NOD-*scid IL2rγ*^{null} mice.

TNFα Promotes Human $CD3^+$ T-Cell Development in NOD-*scid IL2rγ*^{null} Mice Engrafted with Cultured Human UCB. The inability of cultured UCB to generate $CD3^+$ T cells in NOD-*scid IL2rγ*^{null} mice suggests that 1) T-cell progenitors fail to survive in culture, 2) an inhibitory component(s) restricting T-cell development is present, or 3) a factor required for the generation of human $CD3^+$ T cells in the host is absent. One critical factor may be TNFα, as it has been reported that TNFα promotes the *in vivo* generation of T cells in NOD-*scid* mice engrafted with human HSC (46).

Human $CD45^+$ Cell Engraftment in TNFα-Treated NOD-scid IL2rγ^{null} *Mice Transplanted with Cultured Human UCB.* We next determined whether TNFα would enhance the ability of cultured UCB to generate T cells. NOD-*scid IL2rγ*^{null} mice pretreated with TNFα generated levels of human $CD45^+$ cells in the blood (3.1 ± 0.8%; 7.1 ± 2.4×10^4/ml, $n = 32$) equivalent to mice not given TNFα (4.4 ± 1.1%; 10.0 ± 0.2×10^4/ml, $n = 27$, P = N.S., Fig. 2). Levels of human $CD45^+$ cells in the bone marrow (23.3 ± 4.4%; 6.5 ± 1.3×10^6, $n = 26$) of TNFα treated mice were also equivalent to that in non-TNFα-treated mice (25.6 ± 4.7%; 4.8 ± 1.0×10^6, $n = 19$, P = N.S.).

The percent of $CD45^+$ cells in the spleen of mice pretreated with TNFα (38.6 ± 6.2%, $n = 11$) was significantly greater than that in mice not treated with TNFα (16.2 ± 4.2%, $n = 12$, $P < 0.01$). There was also a difference in cell numbers in non-TNFα-treated mice (2.6 ± 0.9×10^6) and TNFα-treated mice (7.3 ± 1.8×10^6, $P < 0.025$). Non-TNFα-treated mice displayed undetectable (<0.05%, $n = 22$) levels of human $CD45^+$ cell engraftment in the thymus whereas TNFα treated mice had readily detectable human $CD45^+$ cells (47.9 ± 10.7% and 8.6 ± 2.3 × 10^7, $P < 0.001$, Fig. 2).

Thymocytopoiesis and Human $CD3^+$ T-Cell Development in NOD-*scid IL2rγ*^{null} Mice Treated with TNFα and Transplanted with Cultured Human UCB. *Thymus.* In contrast with non-TNFα-treated mice that generated no human thymic engraftment, mice treated with TNFα developed $CD3^+$ T cells in the thymus (Fig. 3). The human CD3 thymocytes displayed a normal distribution

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were analyzed by immunohistochemistry. Thymic sections of TNFα-treated mice contained human $CD45^+$ cells (A), which were localized predominantly in the subcapsular region. Many of these $CD45^+$ cells were $CD5^+$ (B) and expressed either $CD4^+$ (C) and/or $CD8^+$ (D). In addition, $CD1a^+$ thymocytes were detected residing in the cortex of the thymus (E). TdT^+ (F) and $CD99^+$ (G) thymocytes were also present, as were numerous human $CD45^+$ cells that expressed the proliferation antigen Ki-67 (H).

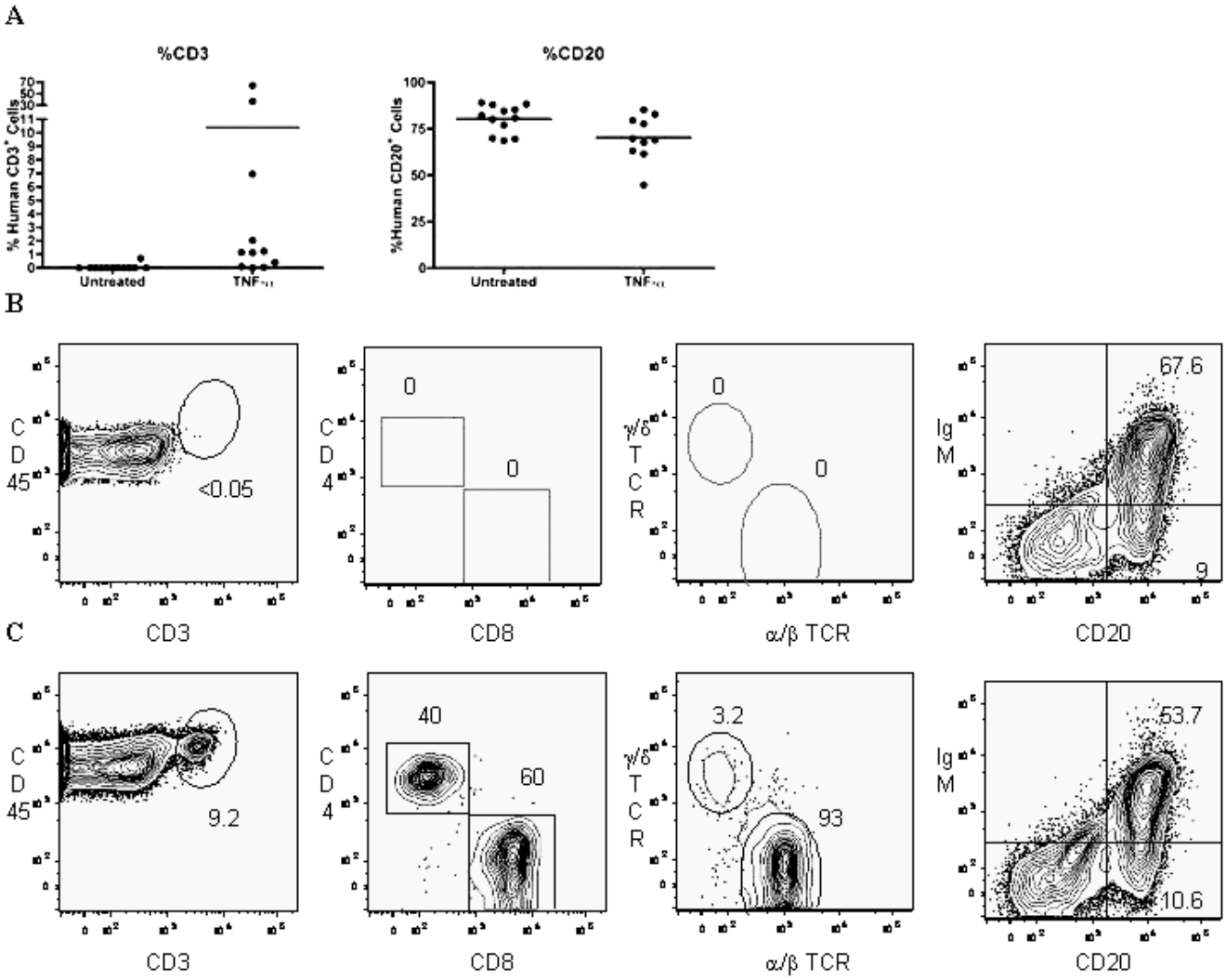

Figure 5. Human CD45$^+$ cell development in the spleen of non-TNFα-treated and TNFα-treated NOD-*scid IL2rγ^{null}* mice twelve weeks after transplantation of cultured human UCB. Irradiated non-TNFα-treated and TNFα-treated NOD-*scid IL2rγ^{null}* mice were transplanted with 1×10^6 cultured CD34$^+$ human UCB cells. Twelve weeks later, spleen cells were analyzed by flow cytometry. (A) Percentage of human CD3$^+$ and CD20$^+$ cells in the spleen. Representative flow cytometry profiles of human cell development in (B) non-TNFα-treated and (C) TNFα-treated NOD-*scid IL2rγ^{null}* mice. Lineage markers expressed as percent of human CD45$^+$ cells. Human CD4 and CD8 percentages are expressed as percent of human CD3$^+$ cells.

of immature double-positive CD4$^+$CD8$^+$ thymocytes and mature single-positive CD4$^+$ and CD8$^+$ T cells. Histological analysis of thymic sections of TNFα-treated mice supports the flow cytometry data (Fig. 4).

Spleen. In contrast to non-TNFα-treated mice in which only very low levels of splenic CD3$^+$ T cells were detected, CD3$^+$ T cells were readily detected in the spleen of mice treated with TNFα (Table 4 and Fig. 5, $P < 0.05$). The CD3$^+$ T cells were comprised of mostly TCRα/β$^+$ cells (90.2 ± 3.7%) with low levels of TCRγ/δ$^+$ cells (2.6 ± 0.6%). The splenic CD3$^+$ population consisted of approximately equal percentages of single positive CD4$^+$ (42.4 ± 7.3) and CD8$^+$ (56.1 ± 6.9) T cells (Fig. 5). Treatment with TNFα did not alter the percent or number of CD20$^+$ B cells.

Splenic sections of TNFα-treated mice displayed human CD45$^+$ cells predominantly in the white pulp (Fig. 6). Human CD20$^+$ B cells as well as human CD5$^+$ T cells were also identified. Human B cell maturation was documented by the presence of human CD138$^+$ plasma cells, which contain kappa and lambda light chains (Fig. 6).

Function of Human T and B Cells in TNFα-Treated NOD-*scid IL2rγ^{null}* Mice Engrafted with Cultured Human UCB. To determine whether engrafted B cells were functional, we first quantified the concentrations of human Igs in the serum of mice engrafted 12 weeks earlier with cultured UCB. Levels of human IgM ranged from ~50 μg/ml to >250 μg/ml and levels of human IgG ranged from ~25 μg/ml to >250 μg/ml. The level of both IgG and IgM was increased in mice immunized with Pneumovax and treated with BLyS (Fig. 7A). Low but detectable levels of IgM and IgG antibody to serotype 4 and serotype 14 of *Streptococcus pneumoniae* were detected

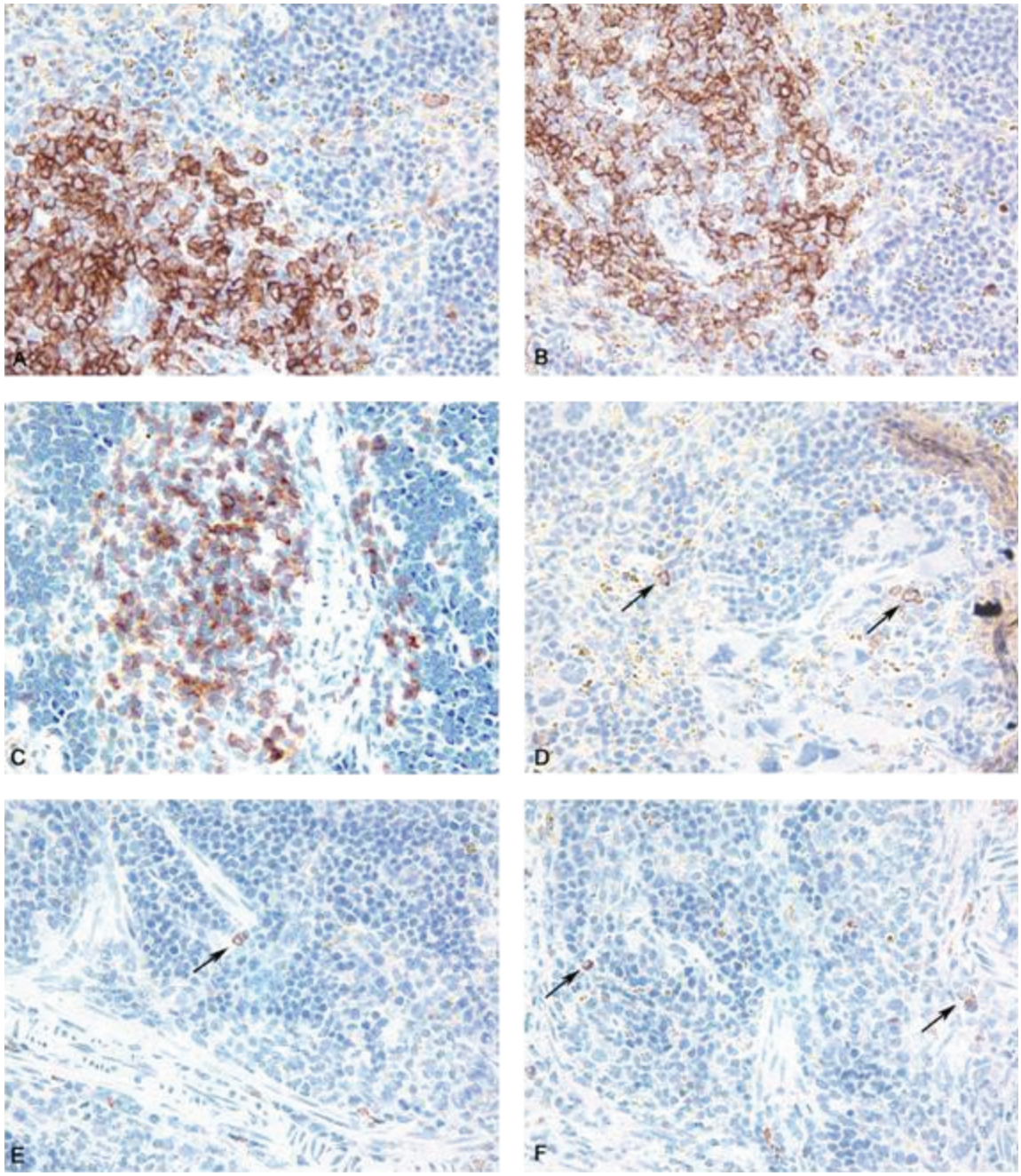

Figure 6. Immunohistochemistry of spleens of TNFα-treated NOD-*scid IL2r*γ^{null} mice twelve weeks after transplantation of cultured human UCB. TNFα-treated NOD-*scid IL2r*γ^{null} mice were transplanted with 1×10^6 cultured $CD34^+$ human UCB cells. Twelve weeks later, the distribution of human lymphocytes in the spleen was determined by immunohistochemistry. Immunoperoxidase staining of human (A) $CD45^+$, (B) $CD20^+$, (C) $CD5^+$, (D, arrows) $CD138^+$, (E, arrows) $kappa^+$ and (F, arrows) $lambda^+$ cells in spleens of TNFα-treated NOD-*scid IL2r*γ^{null} mice.

following immunization with Pneumovax, and administration of recombinant human BLyS in mice boosted with Pneumovax significantly increased the concentration of IgM antibody to both serotypes (Fig. 7B).

Splenocytes obtained from TNFα-treated mice were cultured in the presence of human anti-CD3, anti-CD28, and anti-CD49d mAb for 64 hours. When adjusted for the number of human $CD3^+$ cells in the cultures, the proliferation of human $CD3^+$ cells in the spleen of engrafted TNFα-treated NOD-*scid IL2r*γ^{null} mice was only slightly lower than that of freshly isolated human T cells (Fig. 8A). Finally, to determine if the functional T and B cells could collaborate to generate a T-dependent antibody response, mice were immunized with tetanus and boosted in the presence of recombinant human BLyS. Anti-tetanus antibody was detected in the serum of 2/7 of the immunized mice and 2/3 of mice boosted in the presence of BLyS (Fig. 8B).

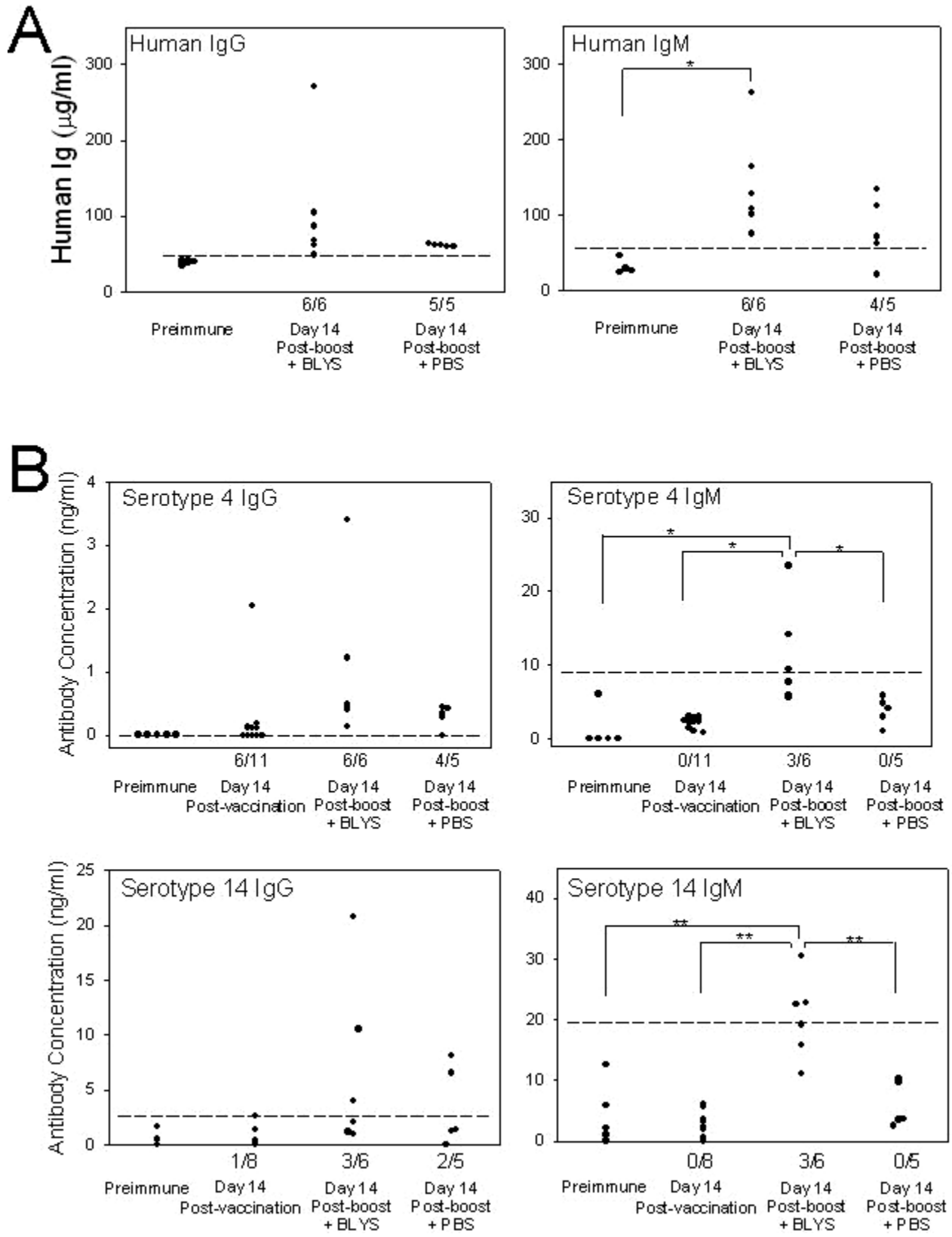

Figure 7. Human immunoglobulin levels and T-independent immune responses in TNFα-treated NOD-*scid IL2rγ*^{null} mice twelve weeks after transplantation of cultured human UCB. TNFα-treated NOD-*scid IL2rγ*^{null} mice were transplanted with 1×10^6 cultured CD34$^+$ human UCB cells. Represented by the dashed line in each panel is the upper limit of the mean of pre-immune sera plus 3 standard deviations. All values above this limit were considered ''responders.'' The treatment group and the number of responders for each treatment group are shown at the bottom of each panel. Each symbol represents an individual animal. Panel A: Human IgG and IgM antibodies prior to and following Pneumovax

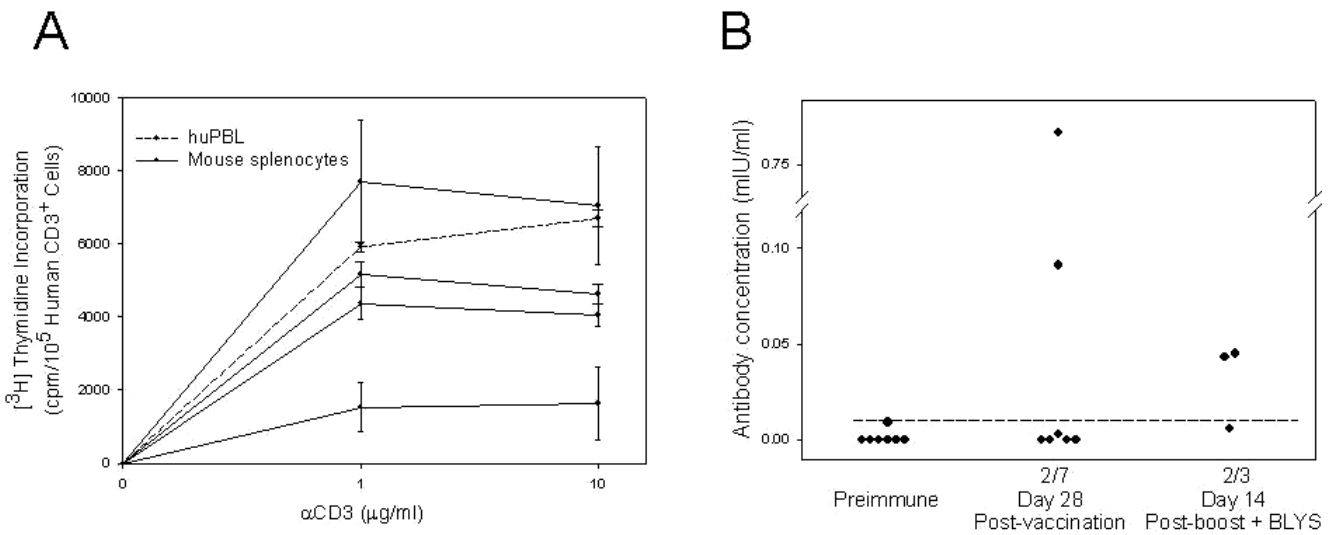

Figure 8. Human CD3 proliferation and human T-dependent immune responses in TNFα-treated NOD-*scid IL2rγ*^{null} mice twelve weeks after transplantation of cultured human UCB. TNFα-treated NOD-*scid IL2rγ*^{null} mice were transplanted with 1×10^6 cultured CD34$^+$ human UCB cells. Panel A: Proliferation of human CD3 T cells following *ex vivo* stimulation with anti-CD3 twelve weeks after transplantation. Shown are results of triplicate cultures of spleen cells from individual mice displayed as mean ± SD. Panel B: Human IgG antibodies to tetanus following immunization with tetanus toxoid. Each symbol represents an individual animal. Represented by the dashed line in each panel is the upper limit of the mean of pre-immune sera plus 3 standard deviations. All values above this limit were considered "responders." The treatment group and the number of responders for each treatment group are shown at the bottom of the panel. Each symbol represents an individual animal.

Discussion

We have investigated the functional potential of human cord blood CD34$^+$ cells expanded in culture for two weeks to generate *in vivo* a multi-lineage human hematopoietic system and a functional human immune system. Cultured human UCB generated myeloid, erythroid, and lymphoid-lineage cells in NOD-*scid IL2rγ*^{null} mice. Administration of TNFα facilitated the generation of human T cells. The immune system of these engrafted mice was functional as assessed by the presence of human Ig, and the ability to generate antibodies in response to immunization with either T-independent or T-dependent antigens.

Multiple sources of human HSC are now used for clinical transplantation, including mobilized peripheral blood hematopoietic stem cells, bone marrow, and umbilical cord blood (47, 48). However, cord blood is emerging as the HSC source of choice due to the low cellular immunity of the passenger leukocytes, and the ease of identifying HLA-matches due to the observation that cord blood can be transplanted with a greater HLA disparity than can HSC from mobilized stem cells or bone marrow (8, 11). One remaining barrier is that clinical transplantation of human UCB cells into adults is limited by the low yield of CD34$^+$ cells from a single cord. Currently, adult transplantation of the required CD34$^+$ cell dose necessitates the pooling of multiple cords, increasing the risk of GVHD as well as the need for matching to multiple donors [for review, see (49, 50)]. Many approaches for *ex vivo* expansion of cord blood cells have been reported (21–25, 51–55). In the culture system used in our studies, a substantial proportion of the cells phenotypically appeared to retain multi-lineage potential. To assess their *in vivo* potential, we adoptively transferred the cells into conditioned NOD-*scid IL2rγ*^{null} mice. The use of this stock of mice was used based on 1) earlier observations showing that human HSC engraft better than in NOD-*scid* mice (36, 56) and 2) our initial experiment comparing directly the ability of cultured HSC to engraft in these two strains. Although NOD-*scid IL2rγ*^{null} mice is the strain we chose for these *in vivo* analyses of human HSC, other strains of immunodeficient mice based on mutations in the IL-2 receptor gamma chain have been reported (34). We recently compared engraftment in BALB/c-*Rag1*^{null} *IL2rγ*^{null} mice with that of NOD-*scid IL2rγ*^{null} mice, and have determined that NOD-*scid IL2rγ*^{null} mice are much better recipients of human HSC (TP, unpublished observations). Moreover, additional modification of NOD-*scid IL2rγ*^{null} mice is currently underway, including the introduction of transgenes expressing human HLA (57).

Previous studies comparing the *in vivo* engraftment kinetics and developmental potential of cultured UCB cells in immunodeficient mice have reported that cultured cells exhibit delayed engraftment (32, 33). In our study, cultured HSC exhibited STRC capacity as evidenced by their ability to generate human myeloid and lymphoid cells within 3 weeks after transplantation. The cultured cells also exhibited

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immunization in the presence or absence of BLyS treatment. Panel B: Human IgG and IgM antibodies to serotype 4 and 14 following Pneumovax immunization in the presence or absence of BLyS treatment. Each symbol represents an individual animal. *$P < 0.05$, **$P < 0.01$.

LTRC capacity and serial transfer and engraftment of the bone marrow into secondary NOD-*scid IL2rγ*^{null} recipients suggests the maintenance of true HSC in the cultured population.

Few human T cells are observed following the injection of cultured HSC into NOD-*scid* mice (23, 32, 33). In those studies, the percentages of human T cells were low, and the possibility of contaminating human T cells in the injected cell population was not excluded. In one study, cells recovered from the mice required *ex vivo* expansion in the presence of PHA and IL-2 to detect T cells (23). We also observed that few cultured HSC develop into T cells in immunodeficient mice.

We hypothesized that the cultured cells may fail to produce TNFα, which increases T-cell lineage commitment and differentiation of human stem cells in NOD-*scid* mice transplanted with HSC (46). Administration of exogenous TNFα prior to transplantation of cultured HSC augmented human thymus cell engraftment. These thymocytes migrated to the peripheral tissues leading to the generation of mature CD4 and CD8 T cells. How TNFα augments T-cell development is not known, but it has been reported that TNF enhances human T-cell generation in a xenogeneic fetal thymus organ culture, suggesting a direct effect on the engrafted stem cells (58). However, experiments where cultured CD34$^+$ cells were treated with TNFα prior to injection into NOD-*scid IL2rγ*^{null} mice, no increase in T-cell development was observed (LJG and KB, unpublished observations). Alternatively, our data are consistent with the interpretation that TNF could have an indirect effect on the host environment in which the stem cells mature, possibly by activation of innate immunity and the induction of cytokines (59). Multiple cytokines have been shown to enhance human HSC engraftment and differentiation in immunodeficient mice [recently reviewed in (57)].

The human immune system generated by cultured HSC in NOD-*scid IL2rγ*^{null} mice was functional. Human IgG and IgM were detected in the circulation, and the mice were able to generate both T-independent and T-dependent antibodies in response to immunization with Pneumovax and tetanus, respectively. We note, however, that we only assayed the antibodies generated to 2 of the 23 serotypes of Pneumovax. We chose these two serotypes to analyze because they are two serotypes shared by both the pneumovax23 adult vaccine and the protein conjugated childhood vaccine (38). Also, they elicit different responses in young versus older individuals: the response to serotype 4 is down in aged individuals whereas the response to serotype 14 remains high (60). The ability to generate antibodies following immunization confirms the phenotypic and histological data showing that T cells, B cells, myeloid, and dendritic cells of human origin were generated. Equally important is that the allogeneic immune system that developed from the HSC in the expanded cord blood is tolerant to host, and could lead to the induction of donor-specific tolerance (61, 62) or for the treatment of autoimmune disorders (63–66).

In summary, human CD34$^+$ UCB cells expanded for two weeks in culture engraft and differentiate into multiple hematopoietic lineages in NOD-*scid IL2rγ*^{null} mice and generate a functional human immune system. *Ex vivo* expansion of UCB CD34$^+$ stem cells may be a promising alternative to pooling multiple cords for adult hematopoietic stem cell transplantation.

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