Enhanced Immune System Regeneration in Humans Following Allogeneic or Autologous Hemopoietic Stem Cell Transplantation by Temporary Sex Steroid Blockade

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Abstract

Purpose: To determine if temporarily blocking sex steroids prior to stem cell transplantation can increase thymus function and thus enhance the rate of T cell regeneration.

Experimental Design: This was a pilot study of luteinizing hormone–releasing hormone agonist (LHRH-A) goserelin given 3 weeks prior to allogeneic or autologous hemopoietic stem cell transplantation and administered up to 3 months posttransplantation. Patients (with or without LHRH-A administration) were assessed from 1 week to 12 months posttransplantation for multiple immunologic variables by flow cytometry (particularly naïve T cells), quantitative PCR to assess T-cell receptor excision circle levels (as a correlate of thymus function), CDR3 length analysis to determine the variability of the TCR repertoire, and *in vitro* assays to determine functional T cell responses.

Results: LHRH-A administration prior to stem cell transplantation significantly increased neutrophil and lymphocyte numbers within the first month of posttransplantation. Most importantly, total and naïve $CD4^+$ T cell regeneration together with T-cell receptor excision circle production, T cell repertoire regeneration, and peripheral T cell function were also significantly enhanced at multiple time points posttransplant. In addition, an increase in disease-free survival ($P = 0.04$) was seen in the autologous setting. Although LHRH-A administration increased T cell responses *in vitro*, it did not exacerbate graft-versus-host disease in the allogeneic setting.

Conclusions: This study provides an important new approach to the improvement of immune reconstitution in patients undergoing hemopoietic stem cell transplantation and may have generic applications in many T cell–based disorders.

The ability to regenerate the peripheral T cell pool following hemopoietic stem cell transplantation (HSCT) is critically dependent on adequate thymic function (1). However, the thymus undergoes age-associated atrophy, predominantly due to an increase in circulating sex steroids from puberty (2). Although persistent thymic function is evident with age (2, 3), there is a profound decrease in T cell output as measured by peripheral blood T-cell receptor excision circle (TREC) levels (3, 4), resulting in a reduction in T cell diversity within the peripheral pool (5). These changes are manifested as a decline in acquired immune responses with age (2).

In healthy individuals, homeostatic mechanisms maintain the peripheral T cell pool (6)—it is generally only following periods of severe T cell depletion that the decline in T cell output by the aged thymus becomes detrimental. Indeed, regeneration of naïve $CD4^+$ T cells is significantly impaired in adults compared with children (7, 8). A means to hasten the rate of immune system regeneration is imperative for improving patient survival posttransplantation. Previous studies have considered the use of factors such as interleukin 7 (IL-7; refs. 9–11), keratinocyte growth factor (12), and Flt3-L (13) in preclinical trials, all of which promote T cell production and survival. Murine experiments using keratinocyte growth factor or Flt3-L have also shown enhanced thymic-dependent and -independent regeneration of T cells following bone marrow transplantation (12, 13), but it remains to be seen how this translates to the clinic. Administration of IL-7 has shown clear enhancement of peripheral T cell reconstitution following murine bone marrow transplantation (9–11), but this is predominantly through thymic-independent means. Furthermore, the effect this has on graft-versus-host disease (GVHD) incidence remains controversial (10, 14); thus, potentially limiting the use of IL-7 in the clinic.

Due to the predominant role of sex steroids in causing thymic atrophy with age, we have shown that their blockade in mice results in full reversal of thymic atrophy and enhanced regeneration of T cells following T cell depletion (2, 15).

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Table 1. Patient pretransplant characteristics

	Patient group			
	Allogeneic control	**Allogeneic LHRH-A**	**Autologous control**	**Autologous LHRH-A**
Patient no.	22	20	21	20
Sex (male/female)	15/7	12/8	12/9	11/9
Median age, y (range)	39 (17-57)	37 (21-53)	58 (22-68)	56 (31-69)
Diagnosis				
Acute leukemia	11	13	1	0
Chronic leukemia	5	0	0	0
Myelodysplasia	0	1	0	0
Lymphomas	1	4	7	9
Multiple myeloma	2	1	13	11
Aplastic anemia	3	1	0	0
Conditioning				
Cyclophosphamide/ATG	1	1		
Cyclophosphamide/TBI	13	10	1	0
Cyclophosphamide/TBI/ATG	1	2	—	—
Fludarabine/Melphalan	5	7	—	—
Fludarabine/Melphalan/ATG	0	2	—	—
Busulfan/Melphalan	1	0	—	—
Melphalan	—	—	13	12
LACE	1	0	7	7
BEAM			0	1
Posttransplantation modulators				
Intragam	16	11	—	—
G-CSF	11	9	—	—
GVHD prophylaxis				
Remission status at transplant				
Complete response	4	5	5	6
Partial response	2	1	12	7
Primary refractory	4	2	0	0
Progressive disease	5	1	3	1
Stable disease	1	0	0	3
Relapse refractory	1	0	1	0
Relapse untested	5	11	0	3
Stem cell source				
Blood	16	19	21	20
Marrow	6	1	0	0
Donor				
HLA identical related	12	12	—	—
HLA matched unrelated	7	7	—	—
Partially matched	3	1	—	—

Abbreviations: ATG, antithymocyte globulin; TBI, total body irradiation; LACE, lomustine, etoposide, cytarabine, and cyclophosphamide; BEAM, carmustine, etoposide, cytarabine, and melphalan; G-CSF, granulocyte colony-stimulating factor.

Temporary chemical castration can be achieved using luteinizing hormone–releasing hormone analogues (LHRH-A); widely used in the clinic to treat numerous endocrinologic disorders such as endometriosis (16), precocious puberty (17), prostate cancer (18), and breast cancer (19). Pretreatment using these analogues has also been tested in order to protect against chemotherapy-induced sterility in both males and females undergoing HSCT (20). However, very little work has been done on the beneficial effects of these drugs on the immune system. We have previously shown that prostate cancer patients routinely treated with LRHH-A can significantly increase the numbers of naïve CD4+ and CD8+ T cells in the peripheral blood (2). In the present study, we show a significant enhancement of naïve ($TREC^+$) T cell regeneration following allogeneic or autologous HSCT when LHRH-A was administered prior to transplantation. In addition, the temporary block in sex steroid production resulted in an increase in the peripheral T-cell receptor (TCR) repertoire and functional T cell responses. This study provides an important new approach to the improvement of immune reconstitution in patients undergoing HSC transplantation and may have generic applications in many T cell–based disorders.

Materials and Methods

Patients, disease, and treatment regime. Patient age, sex, disease, and pretransplant regimen are presented in Table 1. This was a nonrandomized pilot study involving patients with HSCT from The Alfred Hospital and the Peter MacCallum Cancer Institute in Melbourne, Australia from 2001 to 2005. For patients who consented to treatment with the LHRH-A goserelin (Zoladex, AstraZeneca), an initial dose of 10.8 mg for men (3 months) and 3.6 mg for women (1 month) was administered 21 days prior to transplantation. For men, an additional 1-month dose (3.6 mg) was given at day 63 post-HSCT and women were given monthly injections of 3.6 mg with the final dose at day 63 (thus, 4 months total with 3 months post-HSCT administration). The doses used were those preapproved for other indications.

In all LHRH-A–treated patients, castrate levels of sex steroids were obtained within the first month of administration. Castrate levels of estradiol were maintained until 6 months post-HSCT in LHRH-A–treated patients, whereas testosterone levels were castrate until 4 months posttransplant, whereby they increased to ~1 ng/mL and were maintained at this level until 6 months posttransplantation (data not shown). They did not return to control levels until 12 months posttransplant. Although testosterone, follicle-stimulating hormone and luteinizing hormone levels were stable in control patients (non–LHRH-A treated), considerable variation due to chemotherapeutic (and natural cyclic) effects was observed in estradiol and progesterone levels in females (data not shown).

All patients were analyzed pretreatment, weekly for 5 weeks after HSCT, then at 2, 3, 4, 5, 6, 9, and 12 months post-HSCT. Ethics approval was obtained from The Alfred Committee for Ethical Research on Humans (trial no. 01/006).

Cell counts and phenotypic analysis

Blood lymphocyte counts were routinely evaluated using a CELL-DYN 1200 (Abbott Laboratories) at each time point post-HSCT. Phenotypic analysis was done on whole blood. Twenty microliters of the appropriate antibody cocktail was added to 200 μL of whole blood and incubated in the dark at room temperature for 30 min. For removal of RBC, 2 mL of fluorescence-activated cell sorting (FACS) lysis buffer (Becton Dickinson) was then added to each tube and incubated for 10 min at room temperature in the dark. Samples were centrifuged at 600_{gmax}, supernatant was removed and cells washed twice in PBS/FCS/Azide. Finally, cells were resuspended in 1% paraformaldehyde for FACS analysis. Samples were stained with fluorochrome-conjugated antibodies to CD19, CD4, CD8, CD27, CD45RA, CD45RO, CD62L, CD56, CD28, αβTCR, γδTCR, CD11b, CD11c, and CD34 (all from PharMingen); Vα24-FITC and Vβ11-PE (Serotec); and CD8β (Dako).

Intracellular cytokine staining. Whole blood (200 μL) was stimulated with soluble purified anti-CD3 (5 μg/mL) and anti-CD28 (10 μg/mL; both from PharMingen) for 6 h at 37°C, 5% CO_2 in capped polypropylene tubes (Becton Dickinson). Brefeldin A (final concentration, 10 μg/mL; Sigma) was added during the final 4 h. Following stimulation, samples were incubated for 15 min at room temperature with 20 μL of 20 mmol/L of EDTA in PBS and transferred to FACS tubes for staining. Samples were surface-stained with anti–CD4-FITC and anti–CD8-CyChrome (PharMingen). Following lysis and permeabilization, cells were stained with anti–IL-4-PE and anti–IFNγ-APC (PharMingen) or the appropriate isotype controls. Unstimulated cells were used as a control for activation.

Sex steroid analysis. At each time point, analysis of testosterone, estrogen, progesterone, luteinizing hormone, and follicle-stimulating hormone levels in plasma/serum was done either in-house or by The Alfred Pathology Services. For in-house analysis, the concentration of testosterone and estradiol was determined using a commercially available competitive solid phase ELISA kit (Alpha Diagnostic International). All standards and samples were tested in duplicate and analyses done according to the instructions of the manufacturer. A standard curve was constructed using varying concentrations of human testosterone or estradiol. For Alfred Pathology analysis, progesterone and testosterone levels were assessed by electrochemiluminescence immunoassay on the E170 (Roche). Luteinizing hormone, follicle-stimulating hormone, and estrogen levels were assessed by immunoassay on the Architect ci8200 (Roche).

Preparation of peripheral blood mononuclear cells. Heparinized peripheral blood was diluted 1:1 with RPMI. Diluted blood was carefully layered over ficoll-hypaque (Sigma) at a ratio of 2:1 blood/ficoll. Tubes were centrifuged at (800_{gmax}) for 25 min at room temperature without brake. The buffy coat layer was removed and washed with RPMI. Tubes were centrifuged at 25°C for 15 min at 600_{gmax}, followed by a second wash at 400_{gmax} for 10 min. Supernatant was removed, cells resuspended in RPMI + 5% AB serum (Sigma), cell counts were done and cells stored in liquid nitrogen until required.

Stimulation assays

For TCR-specific stimulation, 1×10^5 peripheral blood mononuclear cells (PBMC) were incubated on plates coated with purified anti-CD3 (1-10 μg/mL) and anti-CD28 (10 μg/mL). Following plaque formation (48-72 h), 1 μCi of ^{3}H-thymidine was added to each well, and plates incubated for a further 16 to 24 h. Plates were harvested onto filter mats and incorporation of ^{3}H-thymidine was determined using liquid scintillation on a β-counter (Packard-Coulter).

Detection of serum IL-7. The IL-7 concentration in serum samples was analyzed using a commercially available ELISA kit (BioSource International), and analysis was done according to the manufacturer's instructions. Standard curves were constructed using recombinant human IL-7. All standards and samples were tested in duplicate, and the control groups were analyzed in the same assay as the corresponding patient groups to avoid any interassay variation for statistical comparison.

TREC analysis

Cell sorting. Cryopreserved PBMC were rapidly thawed into sterile, polypropylene FACS sorting tubes, washed in FACS buffer containing 1 mmol of EDTA and 1% human serum and centrifuged (600_{gmax}, 5 min, 4°C). Cells were then incubated with anti-CD4 and anti-CD8 for 30 min on ice, washed and fixed by the drop-wise addition of 1 mL of 3% formalin in PBS (with agitation). Samples were incubated for an additional 30 min, washed, and resuspended in 500 μL of FACS buffer for sorting. Samples were sorted using a MoFlo (Dako Cytomation).

DNA isolation. Cells were sorted directly into PCR grade 0.6 mL Eppendorf tubes, centrifuged (8 min, $2{,}500_{gmax}$) and resuspended in Proteinase K digestion buffer (2×10^5 cells/20 μL of a 0.8 mg/mL solution). Samples were incubated for 1 h at 56°C followed by 10 min at 95°C. Lysed samples were stored at -70°C prior to real-time PCR.

Real-time PCR using molecular beacons

Real-time PCR for analysis of TREC content was done as described previously (21). Each 50 μL reaction contained 5 μL of DNA, and the final concentration of each component was as follows: 1× TaqMan buffer A, 3.5 mmol of $MgCl_2$, 0.4 pmol/μL of each primer, and 1.25 units of AmpliTaq Gold DNA polymerase. The primers were sense, 5′-GGATGGAAAACACAGTGTGACATGG-3′ and antisense, 5′-CTGTCA-ACAAAGGTGATGCCACATCC-3′. One cycle of denaturation (95°C for 10 min) was done, followed by 45 cycles of amplification (94°C for 30 s, 60°C for 30 s, and 72°C for 30 s). To normalize for cell equivalents in the input DNA, a separate real-time PCR assay was used to quantify the CCR5 coding sequence, which contained no pseudogenes.

Spectratyping

Cell sorting. PBMC were separated into $CD4^+$ and $CD8^+$ T cell populations using EasySep magnetic separation (Stem Cell Technologies) according to the manufacturer's instructions.

Analysis of T-cell receptor repertoire diversity. Total RNA was extracted using RNeasy kit (Qiagen) according to the manufacturer's instructions and cDNA synthesized using 200 units of Moloney murine leukemia virus reverse transcriptase and random hexamers (Promega). Total cDNA was divided equally to be amplified in a 25 μL reaction with 1 of 24 TCR Vβ sense primers and a TCR Cβ Cy5′-labeled antisense primer (Sigma). The final reaction mixture contained 0.25 mmol of each primer, 0.25 mmol of deoxynucleotide triphosphates, 1 mmol of $MgCl_2$, 2.5 μL of 10× buffer, and 1 unit of Red Hot DNA Polymerase (ABgene). PCR conditions were 95°C for 1 min followed by 30 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 1 min, with a final extension of 72°C for 10 min. PCR primer sequences were as previously described (22). Two microliters of fluorescently labeled PCR product was mixed with 2 μL of formamide loading buffer and run on a 6% denaturing gel at 1,500 V for 10 h on an ALF Express (Pharmacia). Products were analyzed using the Allele links program (Pharmacia).

Spectratype complexity scoring. The complexity of Vβ subfamilies was determined by counting distinct peaks. These were graded on a

Table 2. Posttransplantation clinical results

	Patient group			
	Allogeneic control (n = 22)	Allogeneic LHRH-A (n = 20)	Autologous control (n = 21)	Autologous LHRH-A (n = 20)
aGVHD (grade 2-4)	9	7	—	—
cGVHD (extensive)	10	10	—	—
Alive	11	11	19	18
CR	11	11	8	11
Relapse	0	0	6	2*
Dead				
GVHD	1	0	—	—
Infection	1	1	0	0
GVHD/infection	3	4	—	—
Relapse	5	4	2	2
Relapse/GVHD	1	0	—	—

NOTE: Kaplan-Meier disease-free survival estimate.
*P = 0.04 compared with autologous control group.

score of 0 to 8. Normal complexity was characterized by a Gaussian distribution that reflected the presence of polyclonal cDNA. A score of 0 was given if no subfamilies were present, and 1 for a single peak, etc. The overall spectratype complexity score for each sample was calculated by adding the total number of subfamilies. The complexity score in normal individuals ranges from 121 to 185.

Statistics

For survival and relapse, data were analyzed using Kaplan-Meier correlation (SPSS 11.0). For all immunologic variables, data were analyzed using Mann-Whitney U tests or χ^2 test for independence with GraphPad Instat 3.0 software (Software MacKiev) or SPSS 11.3 (SPSS, Inc.). Where possible, data was stratified according to age, sex, and GVHD status. Multivariate analysis was done using Fisher's exact test (SPSS 11.0).

Results

Clinical outcome of patients given LHRH-A administration prior to HSCT. No difference in survival was observed with LHRH-A treatment (Table 2). However, a significant increase in disease-free survival was observed in the autologous setting with LHRH-A administration prior to HSCT (P = 0.04; Table 2; Fig. 1A). Importantly, LHRH-A administration did not exacerbate GVHD in the allogeneic setting (Table 2).

LHRH-A administration accelerates engraftment post-HSCT. In both the allogeneic and autologous settings, pretransplant administration of LHRH-A enhanced neutrophil numbers at various time points posttransplantation (days 9, 12, and 20 for allogeneic and days 10 to 12 for autologous; Fig. 1B and C). Lymphocyte numbers were significantly increased in the allogeneic group at numerous time points (Fig. 1D). Although not significant, a similar trend was seen for the autologous group (Fig. 1E). The median time to engraftment (>500 neutrophils/μL blood) was 14 days (range, 10-28) in the allogeneic LHRH group compared with 16 days (range, 10-27) for the non–LHRH-A–treated group. In the autologous setting, the median time to engraftment for LHRH-treated patients was 12 days (10-15) compared with 13 days (10-32) for the non–LHRH-A–treated patients.

LHRH-A administration enhances CD4$^+$ T cell regeneration post-HSCT. CD4$^+$ T cell levels remained low for up to 6 months post-HSCT (Fig. 2A and B). Overall, the allogeneic group showed higher levels of CD4$^+$ T cells compared with the autologous group (Fig. 2A and B). LHRH-A administration resulted in a significant increase in CD4$^+$ T cells at 1, 3, 9, and 12 months ($P \leq 0.01$ for 1 and 9 months, and $P \leq 0.05$ for 3 and 12 months) post-HSCT in the allogeneic but not the autologous setting (Fig. 2A and B). In addition, a significant increase in the proportion of patients who returned to pretreatment values in the allogeneic group was seen at 9 months post-HSCT (0% in the controls compared with 50% with LHRH-A treatment; $P \leq 0.05$; Table 3). LHRH-A–treated patients also showed a significantly increased ability to regenerate CD4$^+$ T cells to >400/μL blood in both the allogeneic and autologous groups (42% and 73% for allogeneic LHRH-A treated at 9 and 12 months compared with 0% at both time points for controls and 43% for autologous LHRH-A treated at 12 months compared with 6% for controls; $P \leq 0.05$ and ≤ 0.001, respectively; Table 3). A significantly faster rate of recovery of CD4 cells to 200/μL was also observed in the allogeneic but not in the autologous setting with LHRH-A treatment ($P \leq 0.01$; data not shown). An increased number of allogeneic control patients were given bone marrow as the stem cell source (n = 6 compared with 1 for the LHRH-A–treated group). Because it is well documented that patients receiving bone marrow grafts show delayed CD4$^+$ T cell recovery compared with PBSC (23), we did analyses based on patients receiving only PBSC. A significant difference was still observed at both 9 and 12 months post-HSCT, but not for the earlier time points (data not shown).

LHRH-A administration enhances naïve CD4$^+$ T cell regeneration. Multivariate analysis showed that naïve CD4$^+$ T cell regeneration was dependent primarily on LHRH-A treatment (P = 0.038) and secondly on age and cGVHD status (P = 0.036) in the allogeneic setting (Table 3). In patients <30 years at the time of HSCT, no significant difference was observed in naïve CD4$^+$ T cell (CD45RA$^+$CD45RO$^-$CD62L$^+$) reconstitution (data not shown) but was significant for patients >30 years of age from 5 to 12 months post-HSCT ($P \leq 0.05$; Fig. 2C). A significant increase in naïve CD4$^+$ T cell levels in the autologous LHRH-A group was seen at 12 months post-HSCT ($P \leq 0.05$;

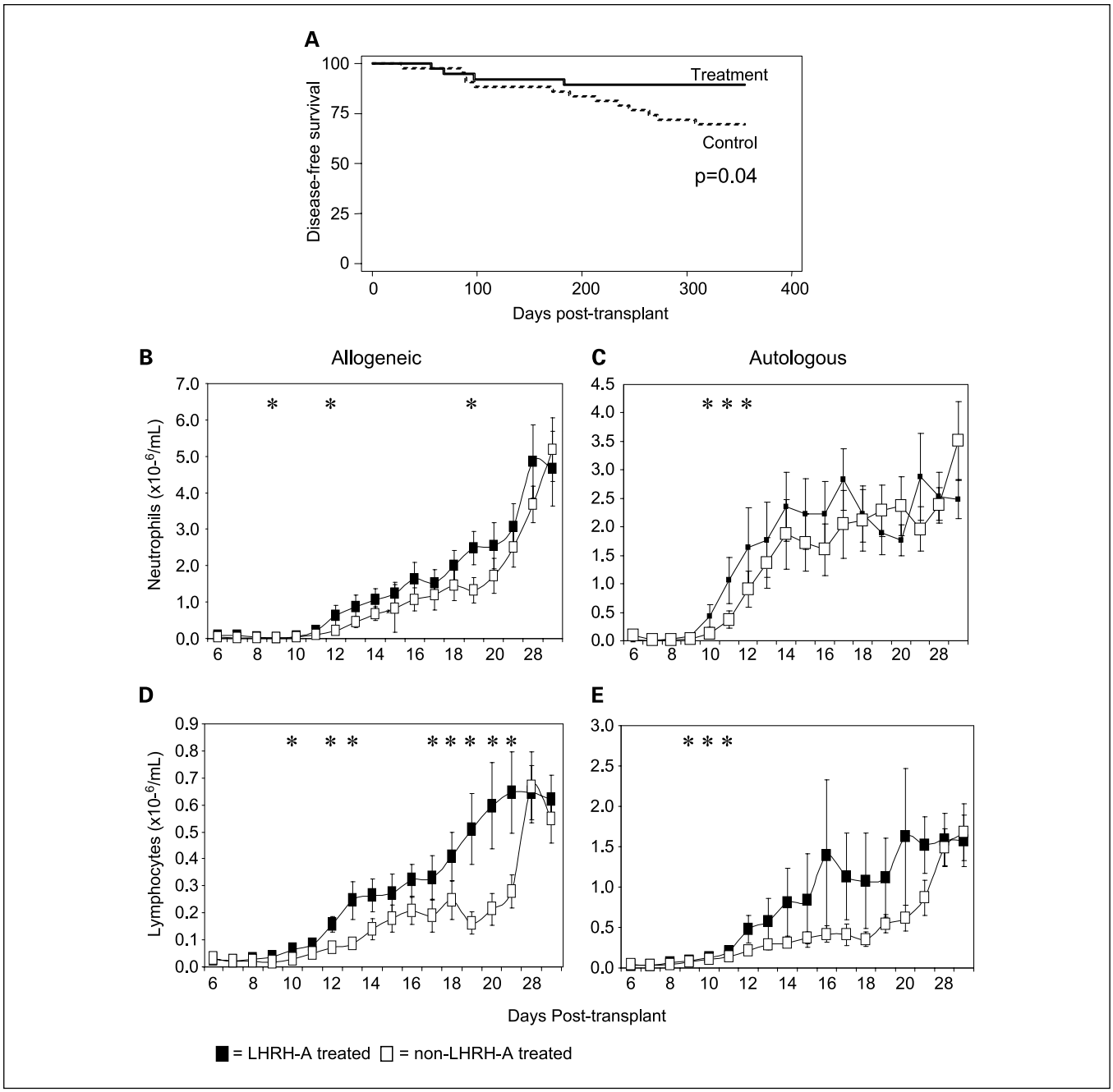

Fig. 1. LHRH-A administration significantly enhances survival and engraftment rates following HSCT. *A,* disease-free survival rates calculated for the length of the trial (12 mo) for autologous transplant recipients. Full blood counts were done on allogeneic (*left*) and autologous (*right*) HSCT patient samples from days 0 to 35 to assess engraftment rates. *B* and *C,* absolute neutrophil counts per milliliter of blood. *D* and *E,* absolute lymphocyte counts per milliliter of blood. Results are expressed as mean ± SE for 12 to 20 patients at each time point (*, $P \leq 0.05$).

Fig. 2D). Interestingly, the LHRH-A–treated group showed an increased ability to return to baseline levels of naïve $CD4^+$ T cells in both the allogeneic and autologous settings (Table 3). This was particularly evident in the allogeneic group with LHRH-A–treated patients showing a significant increase in the ability to regenerate naïve $CD4^+$ T cells >40/μL of blood. In order to assess the influence of the thymus on the regeneration of naïve T cell numbers post-HSCT, PBMC were sorted into $CD4^+$ and $CD8^+$ subsets and analyzed by real-time PCR for TCR-α circles. The increases in naïve T cells correlated highly with increases in TREC levels (e.g., $r = 0.78$ at 12 months for allogeneic LHRH; data not shown). LHRH-A administration resulted in a significant increase in $CD4^+TREC^+$ cells/μL blood at 1 and 9 months post-HSCT ($P \leq 0.05$), and although not statistically significant, was numerically higher at 12 months in the allogeneic setting; TREC levels were higher at both 9 and 12 months post-HSCT in the autologous setting ($P \leq 0.05$ and $P \leq 0.01$, respectively; Fig. 2E and F). The proportion of $TREC^+$ cells (TREC+/100,000 cells) followed a similar pattern with LHRH-A administration significantly increasing levels at 1 and

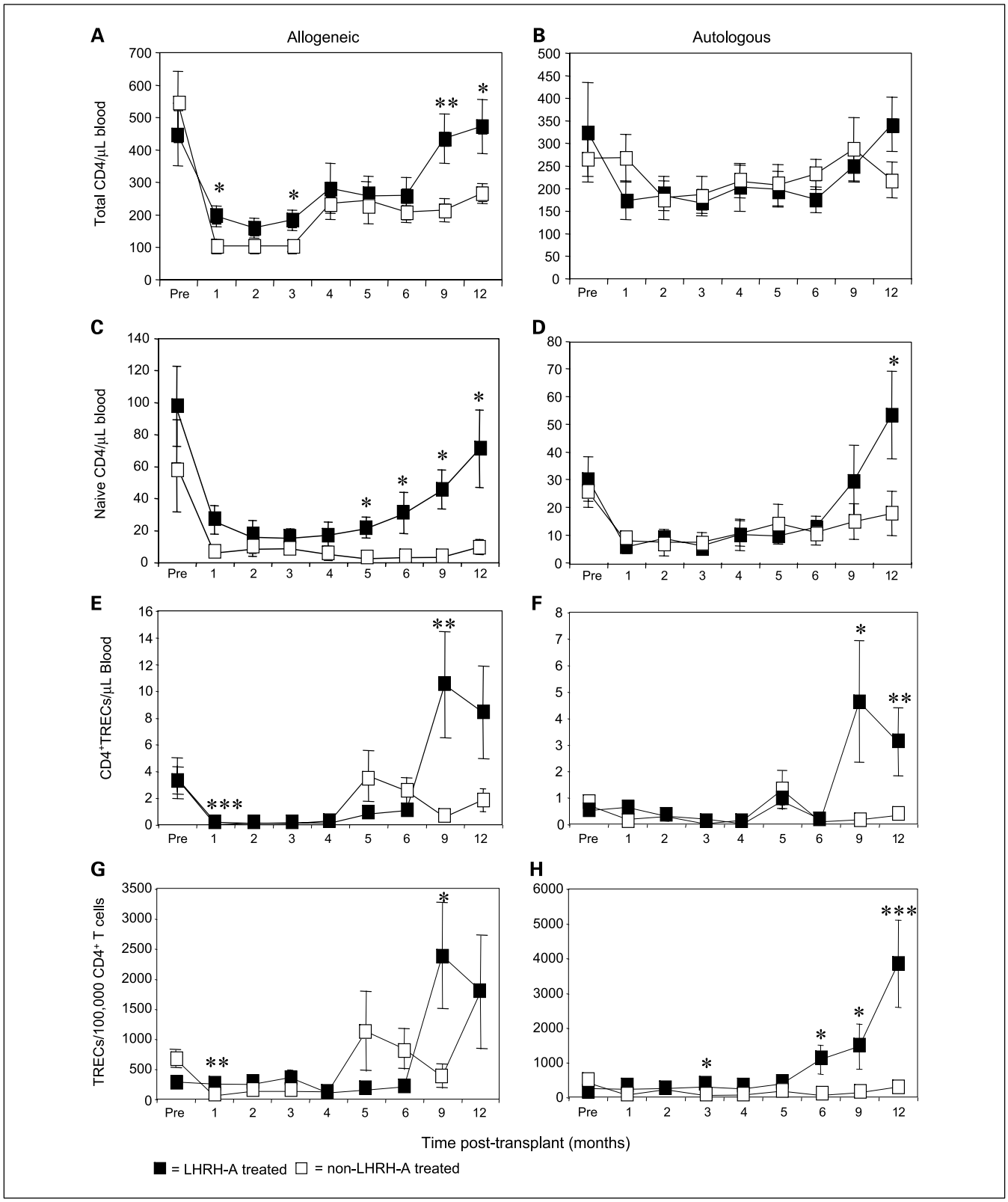

Fig. 2. LHRH-A administration significantly increases total and naïve $CD4^+$ T cell regeneration following HSCT. Whole blood flow cytometry was done on both allogeneic (*left*) and autologous (*right*) patient samples from day 14 to 12 mo posttransplantation. The percentage of positive cells was multiplied by total lymphocyte count to give absolute counts per microliter of blood. *A* and *B,* total CD4 counts per microliter of blood. *C* and *D,* naïve ($CD45RA^+CD45RO^-CD62L^+$) CD4 counts per microliter of blood. The allogeneic groups are presented based on age >30 y. *E* to *H,* PBMC were separated into $CD4^+$ and $CD8^+$ T cells and quantitative PCR was done for alpha circles to assess TREC levels for patient samples from 1 to 12 mo post-HSCT. Levels were standardized using CCR5, which contains no pseudogenes. *E* and *F,* TREC levels expressed as absolute counts per microliter of blood. *G* and *H,* TREC levels expressed as proportion of $TREC^+$ cells/10^{-5} $CD4^+$ T cells. Results are expressed as mean ± SE for 12 to 20 patients at each time point (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$).

Table 3. $CD4^+$ T cell regeneration (percentage of evaluable patients)

	Group	6 mo	9 mo	12 mo
Total return				
Allo	Ctrl	11	0	20
	LHRH	33	50 ($P \leq 0.05$)	45
Auto	Ctrl	41	43	47
	LHRH	56	56	50
Naïve return				
Allo	Ctrl	11	11	20
	LHRH	42	50	45
Auto	Ctrl	18	21	35
	LHRH	38	33	50
TREC/mL return				
Allo	Ctrl	10	22	20
	LHRH	25	40	64 ($P \leq 0.05$)
Auto	Ctrl	50	64	41
	LHRH	63	88	62
Total >400/μL*				
Allo	Ctrl	10	0	0
	LHRH	17	42 ($P \leq 0.05$)	73 ($P \leq 0.001$)
Auto	Ctrl	18 ($P \leq 0.05$)	14	6
	LHRH	0	11	43($P \leq 0.05$)
Naïve >40/μL*, †				
Allo	Ctrl	0	0	0
	LHRH	33	56 ($P \leq 0.05$)	67 ($P \leq 0.01$)
Auto	Ctrl	12	21	13
	LHRH	6	22	50 ($P \leq 0.05$)

NOTE: χ^2 analysis.
*Regeneration of total or naïve $CD4^+$ T cells to >400/μL or 40/μL, respectively.
† Based on patients >30 y of age.

9 months post-HSCT in the allogeneic setting ($P \leq 0.01$ and $P \leq 0.05$, respectively; Fig. 2G) and at 3, 6, 9, and 12 months in the autologous setting ($P \leq 0.05$ for 3 to 9 months and $P \leq 0.001$ for 12 months; Fig. 2H). In the allogeneic setting, the proportion of patients able to regenerate TREC levels to pre-HSCT values was significantly increased with LHRH-A treatment ($P \leq 0.05$ at 12 months post-HSCT; Table 3).

Other cell types that were analyzed in this study included regulatory T cells (Treg), natural killer T cells (NKT), and γδ T cells. Interestingly, no difference in the level of Tregs (CD4+CD25+Foxp3+) was seen between treated and non-treated patients at any time point posttransplantation (data not shown), indicating a preferential bias towards effector T cell production. In contrast, both NKT (assessed by Va24/Vb11 phenotype of invariant cells) and γδ T cells were significantly increased in LHRH-A–treated patients up to 5 months posttransplantation (data not shown).

$CD8^+$ T cell regeneration. In contrast to $CD4^+$ T cell regeneration, $CD8^+$ T cells were regenerated quickly post-HSCT as has been seen previously (7). There was no significant difference in total (Fig. 3A and B) or naïve (Fig. 3C and D) $CD8^+$ T cell recovery seen with LHRH-A treatment, although significance was seen in the allogeneic setting at 3 weeks posttransplantation. Analysis of TREC levels, however, showed findings similar to the $CD4^+$ T cells with LHRH-A significantly increasing $TREC^+CD8^+$ numbers in both the autologous and allogeneic settings (Fig. 3E and F).

T cell repertoire diversity. Analysis of Vβ CDR3 lengths by spectratyping was done on sorted $CD4^+$ and $CD8^+$ T cells to assess the level of diversity of the T cell pool. Analyses were done for Vβ 1 to 24 (Fig. 4A and B) with the overall spectratype complexity score for each sample calculated by adding the total number of subfamilies. The complexity score in normal individuals ranges from 121 to 185. The $CD4^+$ T cell repertoire was already quite diverse by 12 months post-HSCT in both LHRH-A–treated and untreated patients (Fig. 4A and B), with the $CD8^+$ population showing greater clonal dominance (data not shown). Analysis of total peak values showed a significant increase in diversity with LHRH-A administration in the allogeneic transplant setting at 6 and 12 months post-HSCT (for $CD4^+$ T cells; $P \leq 0.01$) and at 6 months for $CD8^+$ T cells ($P \leq 0.05$; Fig. 4C). It is also important to note that the LHRH-A–treated group was well within reference range by 12 months post-HSCT compared with the non–LHRH-A–treated patients. No significant difference in diversity was seen in the autologous setting with both groups showing median $CD4^+$ diversity within reference ranges by 12 months post-HSCT (data not shown).

LHRH-A administration enhances T cell function post-HSCT. The functional responsiveness of patient T cells to anti-CD3/CD28 costimulation after transplant was assessed using ^{3}H-thymidine incorporation. Allogeneic patients who were administered LHRH-A showed a reduced threshold for activation. Thus, at a dose of 5 μg/mL anti-CD3 (CD28 was kept a constant 10 μg/mL), a significant increase in proliferation was observed at 1, 3, and 4 months after transplant in LHRH-A patients compared with controls (Fig. 5A). Similar trends were seen for all other doses of anti-CD3 (data not shown). In contrast, no significant difference was seen for any CD3 dose in the autologous setting (Fig. 5B).

Analysis of IFNγ and IL-4 production following TCR stimulation (αCD3/αCD28) was done by intracellular flow cytometry. No significant difference was observed in IL-4 production by $CD4^+$ or $CD8^+$ T cells (data not shown). In addition, the production of IFNγ was equivalent between the groups but showed a greater increase in the LHRH-A–treated group in the allogeneic setting (Fig. 5C and D). Consistent with these findings, the ratio of IFNγ to IL-4 production also did not differ between groups (data not shown).

It has previously been shown that IL-7 could enhance the production of peripheral T cells (9–11). To test this, we assessed the serum levels of IL-7 using ELISA. No differences were observed between the groups in either the allogeneic or autologous settings (Fig. 5E and F) at any time point analyzed.

Discussion

The ability to regenerate T cell numbers, particularly naïve CD4+, following HSCT is crucial for optimal immune system function and survival of the transplant recipient. Adult transplant recipients can take >2 years to regenerate naïve $CD4^+$ T cell levels due primarily to the atrophy of the thymus with age. In this study, we have shown that blockade of sex steroids prior to HSCT results in significantly increased naïve and $TREC^+$ $CD4^+$ and $CD8^+$ T cell levels.

Within the first month posttransplantation, LHRH-A administration significantly increased neutrophil levels and decreased the time taken to engraft compared with control patients following allogeneic or autologous transplant. A significant increase in total and naïve $CD8^+$ T cells, $CD4^+$ T cells (including $TREC^+$), NKT cells, γδ T cells, and functional T cell responses

were also observed in the first month post-HSCT. It is unclear whether the early increases in the T cell compartment were physiologically important; however, these increases correlated with increases in T cell function early posttransplant. In addition, invariant NKT cells are known to possess potent antitumor (24) and antiviral properties (25), and may be beneficial to the patient early post-HSCT prior to full regeneration of the acquired immune system.

The precise mechanism of action of LHRH-A is unclear. The most obvious effect is to simply reduce sex steroids. In this regard, it is also important to further assess the effects in female patients as chemotherapy-induced hypogonadal output is routinely observed with chemotherapy (20). We did not have enough female patients in this study to critically assess this. However, female patients who were postmenopausal prior to transplant and did not receive the agonist did not show any increases in naïve T cell output, yet some females who were postmenopausal prior to chemotherapy and who received the LHRH-A showed increased naïve T cell levels (data not shown). This indicates that either steroid hormone levels need to be

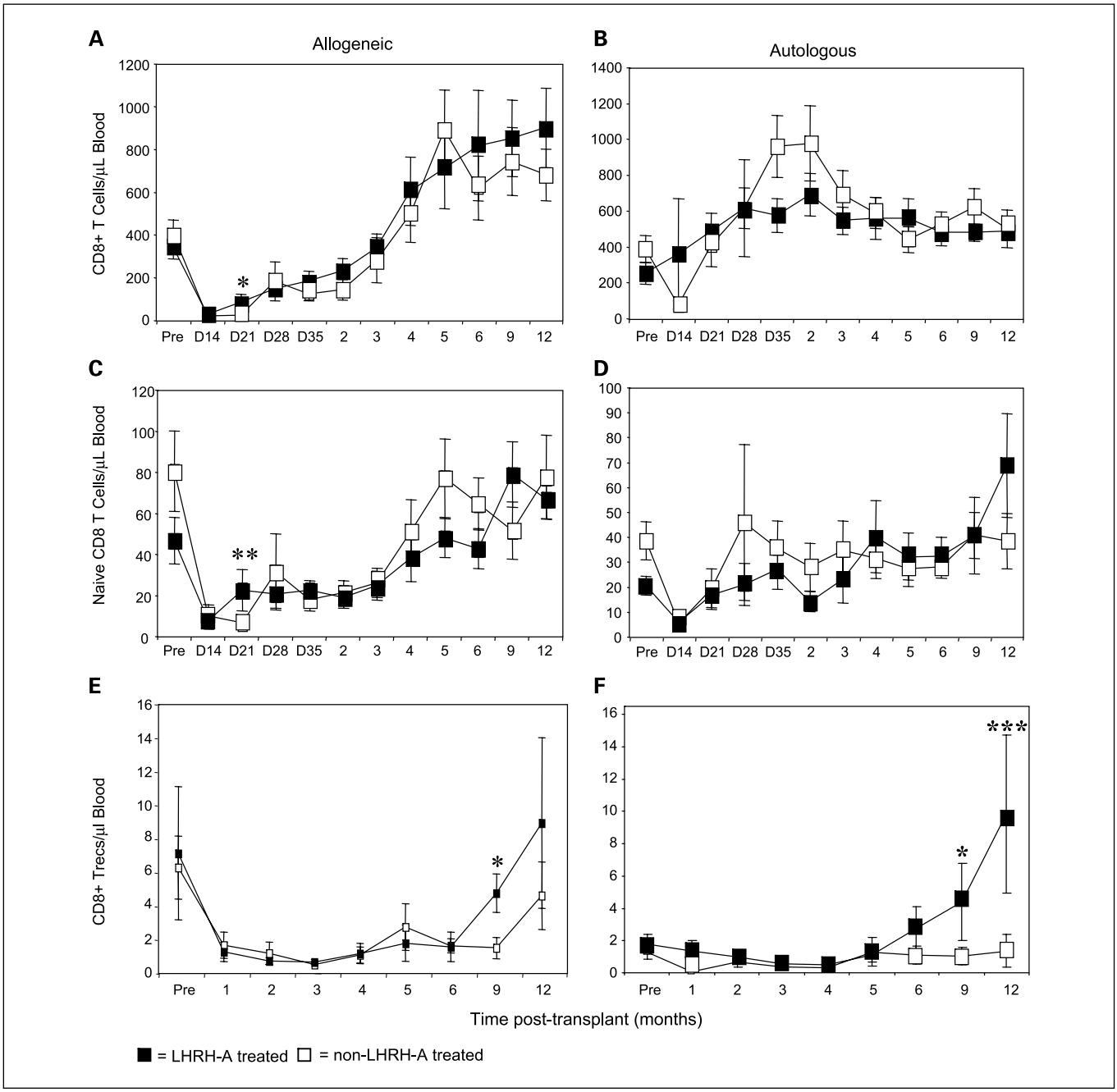

Fig. 3. $CD8^+$ T cell regeneration following HSCT. Whole blood flow cytometry was done on both allogeneic (*left*) and autologous (*right*) patient samples from day 14 to 12 months posttransplantation. The percentage of positive cells was multiplied by total lymphocyte count to give absolute counts per microliter of blood. *A* and *B,* total CD8 counts per microliter of blood. *C* and *D,* naïve ($CD45RA^+CD45RO^-CD62L^+$) CD8 counts per microliter of blood. *E* and *F,* $TREC^+$ cells per microliter of blood. Results are expressed as mean ± SE for 12 to 20 patients at each time point (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$).

decreased globally (similar to LHRH-A administration due to it's direct action on the pituitary; ref. 26) or that LHRH-A may also act directly on the thymus itself, for which there are LHRH receptors (27). We found that LHRH-A administration caused absolute ablation of luteinizing hormone and follicle-stimulating hormone and this was significant compared with control patients up to 6 months post-HSCT in the allogeneic group and 3 months in the autologous group (data not shown).

Arguably, the most important finding of this study was the significant increase in $TREC^+CD4^+$ T cells, which could only be derived from TCR rearrangement in the thymus. Furthermore, LHRH-A–treated patients showed much better "rebound" of $CD4^+$ T cell levels (both total and naïve) and a faster rate of $CD4^+$ T cell recovery to >200/μL. This latter finding has the potential to affect improved clinical outcomes as seen previously (28), and may be evident in the apparently reduced levels of relapse in the LHRH-A–treated autologous transplant recipients in this study. The increase in naïve/$TREC^+$ T cells was coincident with a decrease in peripheral T cell proliferation (detected by Ki-67) as expected: once homeostatic levels have been reached in the peripheral T cell pool, the naïve T cells are able to stabilize and not immediately be induced to become memory T cells to fill up the pool. A further indication of the increased competence for T cell regeneration with LHRH-A treatment are the results from CDR3 spectratyping: LHRH-A–treated patients showed a significant increase in the diversity of the T cell pool in the allogeneic setting, particularly in $CD4^+$ T cells. Interestingly, with LHRH-A–treated patients showing

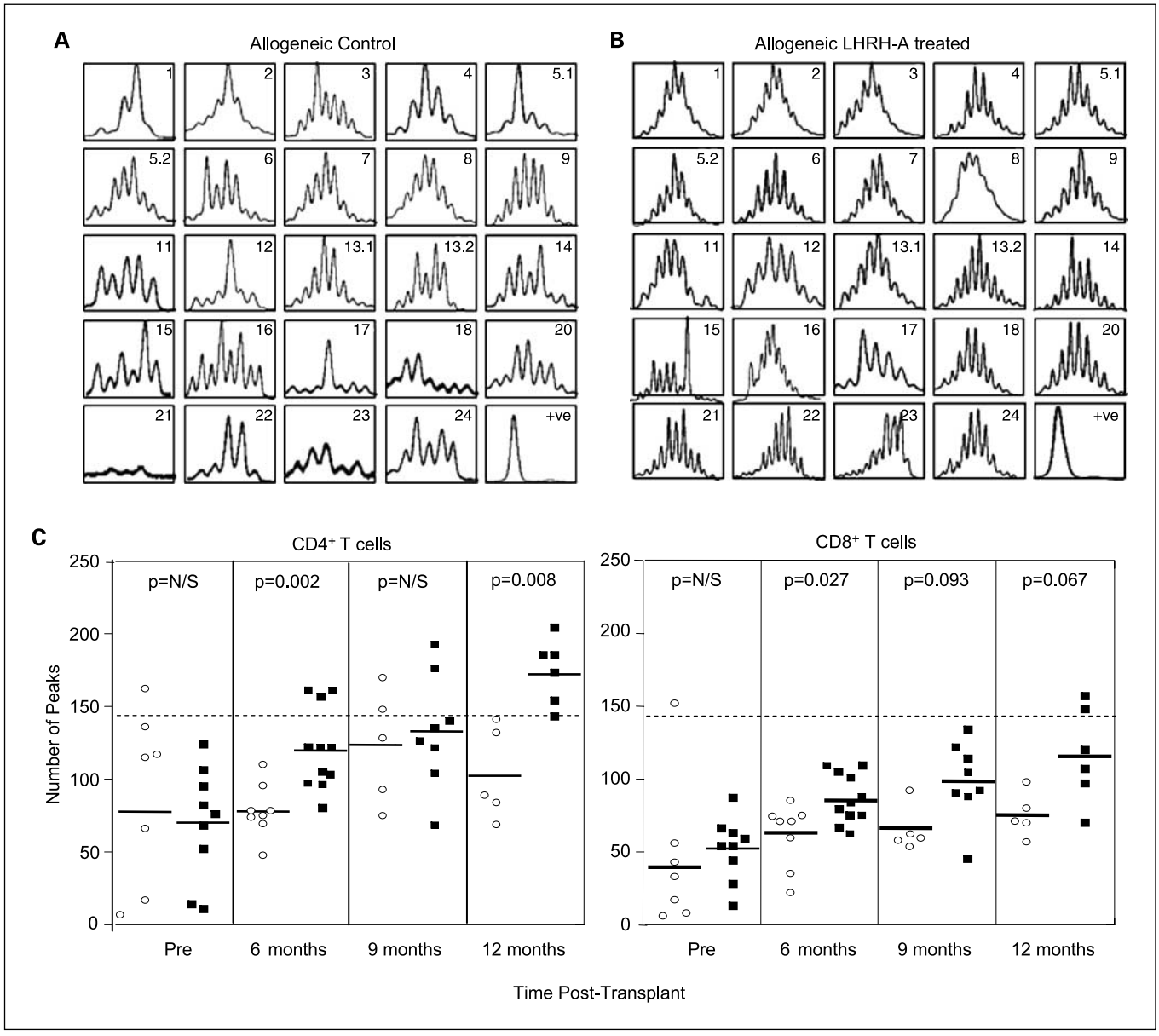

Fig. 4. LHRH-A administration prior to HSCT results in a more diverse peripheral T cell repertoire. PBMC were sorted into $CD4^+$ and $CD8^+$ T cells and CDR3 spectratype analysis done for Vβ1 to 24. *A,* representative histograms of TCRVβ1-24 from an allogeneic control patient ($CD4^+$ sorted cells). *B,* representative histograms of TCR Vβ1-24 from an allogeneic LHRH-A – treated patient ($CD4^+$ sorted cells). *C,* total peak numbers were added for each patient ($CD4^+$ and $CD8^+$). Dotted line, normal control values; solid bar, median of 12 to 20 patients in each group.

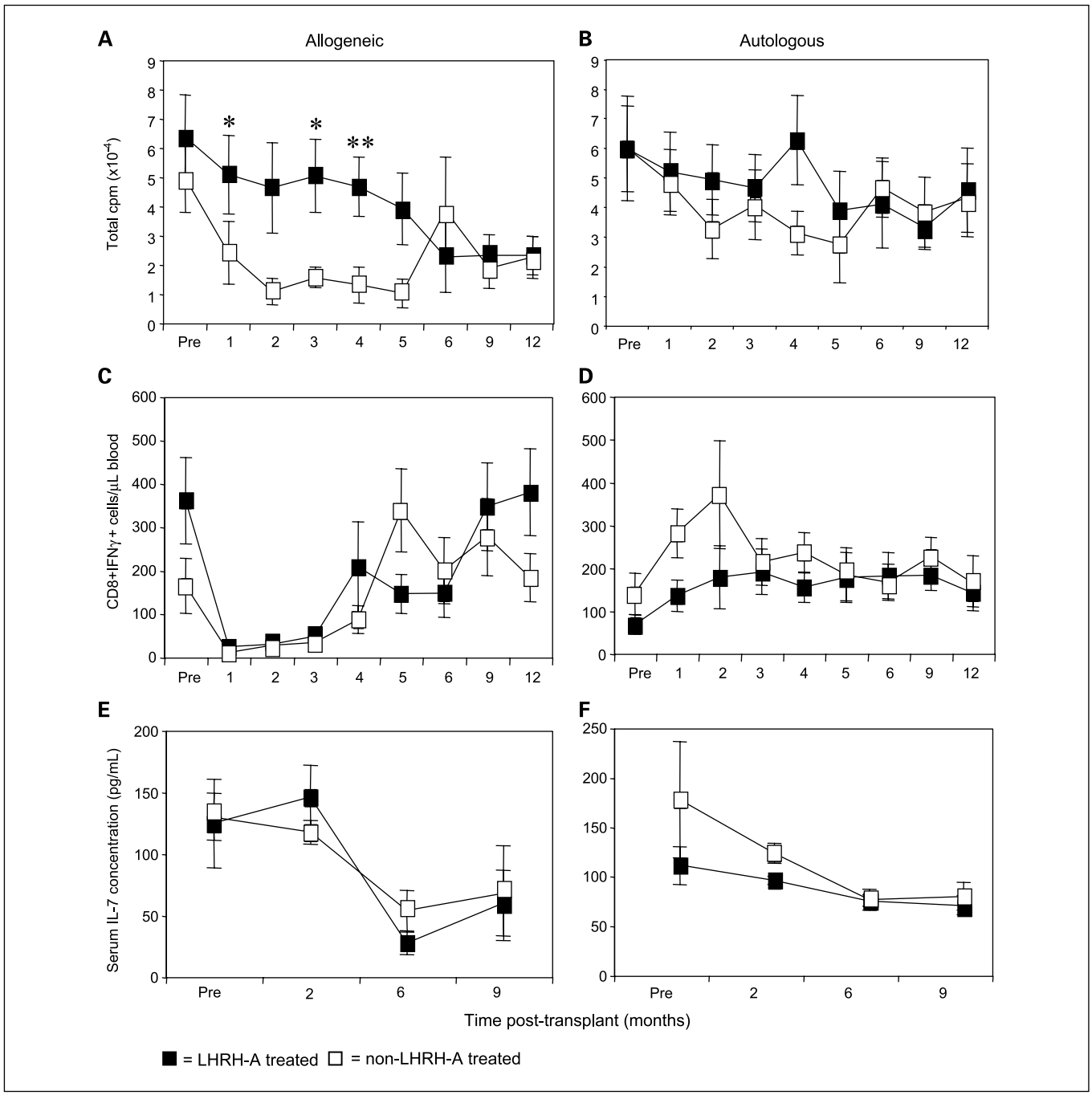

Fig. 5. LHRH-A administration enhances functional T cell responses post-HSCT. *A* to *D,* functional responses of T cells to antigenic stimulation were assessed from 1 to 12 mo posttransplantation in the allogeneic (*left*) and autologous (*right*) settings. *A* and *B,* 1×10^5 PBMC were stimulated with 5 μg/mL of αCD3 and 10 μg/mL of αCD28 for 48 h. ^{3}H-thymidine was added for an additional 16 h and incorporation was assessed using a β-counter. *C* and *D,* whole blood intracellular cytokine detection was done following 6 h of αCD3/αCD28 stimulation. *E* and *F,* to assess the role of IL-7 in the regeneration of naïve T cells posttransplantation, a serum ELISA for IL-7 was done and standardized using recombinant hIL-7. Results are expressed as mean ± SE for 12 to 20 patients at each time point (*, $P \leq 0.05$; **, $P \leq 0.01$).

increases in naïve and TREC$^+$ T cells from 5 to 6 months posttransplantation, there was also a significant increase in diversity within both the CD4$^+$ and CD8$^+$ T cell pools at this time. LHRH-A–treated patients also showed a significant increase in their functional T cell response following direct TCR stimulation. Because the major time points in which functional differences were observed were <6 months post-HSCT, this effect may include direct stimulation by the LHRH-A on the peripheral T cells (29), although we have not been able to show this in peripheral blood stimulation assays *in vitro* (data not shown). This would also be dependent on whether or not the receptors on immune cells undergo similar down-regulation as the pituitary receptors. Regardless, an increase in T cell function following transplantation is critically dependent on thymic function (30), and raises the possibility of an earlier, more successful, vaccination schedule for these patients

posttransplant. Interestingly, the allogeneic transplant recipients seemed to do better on the LHRH-A treatment compared with the autologous group. For instance, both total and naïve CD4+ T cells were regenerated at a faster rate and there were early increases in particular subsets which were not evident in the autologous patients. This is most likely due to the effects of the pretransplant conditioning regime on the bone marrow stem cells: these are only affected in the autologous recipients and it would be expected that this may slow down the rate of development.

Two limitations of this study were the time frame of analysis and the length of LHRH-A administration. These variables warrant further examination—the most logical being to extend the time of administration of LHRH-A, given the fact that the increase in naïve T cells continued in the same period that sex steroids were returning. Extending the LHRH-A administration time may well promote more extensive thymic rejuvenation. Follow-up analysis beyond 12 months would also be important to examine the long-term effectiveness of the treatment. The autologous patients with LHRH-A treatment were just showing significant increases in T and B cell levels at 12 months, again illustrating the importance of long-term follow-up. In addition, no preferential increase in Treg cells was observed, making it unlikely that detrimental effects such as an increase in autoimmunity would occur.[5]

The mechanism underlying thymic regeneration is yet to be fully elucidated. However, our previous work has shown a strong link with sex steroid ablation and the ability of the thymic epithelium to produce thymopoietic cytokines (2). One potential mechanism for the increase in peripheral naïve T cells observed could have been due to increases in circulating IL-7 levels (9–11). However, LHRH-A–treated patients showed no difference in serum IL-7 levels compared with controls, indicating that the increase in naïve T cells (particularly $TREC^+$) is more likely due to increased thymic function. Clinical treatment with IL-7 posttransplantation has shown significant increases in peripheral $CD4^+$ T cell levels (13), but no effect on thymic output. It would be interesting to determine whether the two treatments could synergize to increase both the output and maintenance of $CD4^+$ T cell numbers. The distinct advantage of LHRH-A is that it has been in the clinic for >20 years, with limited side effects. In addition, LHRH-A does not seem to affect the cytokine production by T cells posttransplantation. In fact, we saw quite an early increase in cytokine production which followed the TCR responsiveness assessed by ^{3}H-thymidine incorporation.

In conclusion, we believe that this is a fundamentally important study showing for the first time that it is possible to significantly increase T cell regeneration post-HSCT, thereby potentially reducing the devastating side effects of the transplantation process. A more extensive multicenter trial is currently under way to evaluate this further. This work also has far-reaching potential for more generic immunotherapy such as in HIV patients undergoing antiretroviral treatment or in tumor vaccination strategies.

[5] This is supported by unpublished data on long-term treatment of prostate cancer patients.

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References

1. Heitger A, Greinix H, Mannhalter C, et al. Requirement of residual thymus to restore normal T-cell subsets after human allogeneic bone marrow transplantation. Transplantation 2000;69:2366–73.
2. Sutherland JS, Goldberg GL, Hammett MV, et al. Activation of thymic regeneration in mice and humans following androgen blockade. J Immunol 2005;175:2741–53.
3. Sempowski GD, Gooding ME, Liao HX, et al. T cell receptor excision circle assessment of thymopoiesis in aging mice. Mol Immunol 2002;38:841–8.
4. Douek DC, McFarland RD, Keiser PH, et al. Changes in thymic function with age and during the treatment of HIV infection. Nature 1998;396:690–5.
5. Naylor K, Li G, Vallejo AN, et al. The influence of age on T cell generation and TCR diversity. J Immunol 2005;174:7446–52.
6. Berzins SP, Boyd RL, Miller JF. The role of the thymus and recent thymic migrants in the maintenance of the adult peripheral lymphocyte pool. J Exp Med 1998;187:1839–48.
7. Mackall CL, Fleisher TA, Brown MR, et al. Age, thymopoiesis, and $CD4^+$ T-lymphocyte regeneration after intensive chemotherapy. N Engl J Med 1995;332:143–9.
8. Weinberg K, Blazar BR, Wagner JE, et al. Factors affecting thymic function after allogeneic hematopoietic stem cell transplantation. Blood 2001;97:1458–66.
9. Mackall CL, Fry TJ, Bare C, et al. IL-7 increases both thymic-dependent and thymic-independent T-cell regeneration after bone marrow transplantation. Blood 2001;97:1491–7.
10. Alpdogan O, Schmaltz C, Muriglan SJ, et al. Administration of interleukin-7 after allogeneic bone marrow transplantation improves immune reconstitution without aggravating graft-versus-host disease. Blood 2001;98:2256–65.
11. Broers AE, Posthumus-van Sluijs SJ, Spits H, et al. Interleukin-7 improves T-cell recovery after experimental T-cell-depleted bone marrow transplantation in T-cell-deficient mice by strong expansion of recent thymic emigrants. Blood 2003;102:1534–40.
12. Min D, Taylor PA, Panoskaltsis-Mortari A, et al. Protection from thymic epithelial cell injury by keratinocyte growth factor: a new approach to improve thymic and peripheral T-cell reconstitution after bone marrow transplantation. Blood 2002;99:4592–600.
13. Fry TJ, Sinha M, Milliron M, et al. Flt3 ligand enhances thymic-dependent and thymic-independent immune reconstitution. Blood 2004;104:2794–800.
14. Sinha ML, Fry TJ, Fowler DH, et al. Interleukin 7 worsens graft-versus-host disease. Blood 2002;100:2642–9.
15. Goldberg GL, Sutherland JS, Hammet MV et al. Sex steroid ablation enhances lymphoid recovery following autologous hematopoietic stem cell transplantation. Transplantation 2005;80:1604–13.
16. Wang J, Zhou F, Dong M, et al. Prolonged gonadotropin-releasing hormone agonist therapy reduced expression of nitric oxide synthase in the endometrium of women with endometriosis and infertility. Fertil Steril 2006;85:1037–44.
17. Vottero A, Pedori S, Verna M, et al. Final height in girls with central idiopathic precocious puberty treated with gonadotropin-releasing hormone analog and oxandrolone. J Clin Endocrinol Metab 2006;91:1284–7.
18. Dondi D, Festuccia C, Piccolella M, et al. GnRH agonists and antagonists decrease the metastatic progression of human prostate cancer cell lines by inhibiting the plasminogen activator system. Oncol Rep 2006;15:393–400.
19. Pritchard K. Endocrinology and hormone therapy in breast cancer: endocrine therapy in premenopausal women. Breast Cancer Res 2005;7:70–6.
20. Blumenfeld Z. Preservation of fertility and ovarian function and minimalization of chemotherapy associated gonadotoxicity and premature ovarian failure: the role of inhibin-A and -B as markers. Mol Cell Endocrinol 2002;187:93–105.
21. Zhang L, Lewin SR, Markowitz M, et al. Measuring recent thymic emigrants in blood of normal and HIV-1-infected individuals before and after effective therapy. J Exp Med 1999;190:725–32.
22. Gregersen PK, Hingorani R, Monteiro J. Oligoclonality in the $CD8^+$ T cell population. Analysis using a multiplex PCR assay for CDR3 length. Ann NY Acad Sci 1995;756:19–27.
23. Petersen SL, Ryder LP, Bjork P, et al. A comparison of T-, B- and NK-cell reconstitution following conventional or nonmyeloablative conditioning and transplantation with bone marrow or peripheral blood stem cells from human leucocyte antigen identical sibling donors. Bone Marrow Transplant 2003;32:65–72.
24. Crowe NY, Coquet JM, Berzins SP, et al. Differential

anti-tumor immunity mediated by NKT cell subsets *in vivo*. J Exp Med 2005;202:1279–88.

25. Baron JL, Gardiner L, Nishimura S, et al. Activation of a non-classical NKT cell subset in a transgenic mouse model of hepatitis B virus infection. Immunity 2002;16:583–94.

26. Marchetti B, Gallo F, Farinella Z, et al. Luteinizing hormone-releasing hormone is a primary signaling molecule in the neuroimmune network [review]. Ann N Y Acad Sci 1998;840:205–48.

27. Marchetti B, Guarcello V, Morale MC et al. Luteinizing hormone-releasing hormone (LHRH) agonist restoration of age-associated decline of thymus weight, thymic LHRH receptors, and thymocyte proliferative capacity. Endocrinology 1989;125:1037–45.

28. Kim DH, Sohn SK, Won DI, et al. Rapid helper T-cell recovery above 200×10^6/l at 3 months correlates to successful transplant outcomes after allogeneic stem cell transplantation. Bone Marrow Transplant 2006; 37:1119–28.

29. Chen HF, Jeung EB, Stephenson M et al. Human peripheral blood mononuclear cells express gonadotropin-releasing hormone (GnRH), GnRH receptor, and interleukin-2 receptor γ-chain messenger ribonucleic acids that are regulated by GnRH *in vitro*. J Clin Endocrinol Metab 1999;84:743–50.

30. Roux E, Dumont-Girard F, Starobinski M, et al. Recovery of immune reactivity after T-cell-depleted bone marrow transplantation depends on thymic activity. Blood 2000;96:2299–303.