Keratinocyte Growth Factor and Androgen Blockade Work in Concert to Protect Against Conditioning Regimen-Induced Thymic Epithelial Damage and Enhance T-Cell Reconstitution Following Murine Bone Marrow Transplantation

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Short Title: KGF and Lupron Additively Restore T-cells post-BMT
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ABSTRACT
Myeloablative conditioning results in thymic epithelial cell (TEC) injury, slow T-cell reconstitution, and a high risk of opportunistic infections. Keratinocyte growth factor (KGF) stimulates TEC proliferation and, when given pre-conditioning, reduces TEC injury. Thymocytes and TEC express androgen receptors and exposure to androgen inhibits thymopoiesis. In this study, we have investigated whether TEC stimulation via pre-conditioning treatment with KGF and leuprolide acetate (Lupron™), two clinically approved agents, given only prior to conditioning would circumvent the profound TEC and associated T-cell deficiency seen in allogeneic bone marrow transplant (BMT) recipients. Only combined treatment with KGF plus Lupron normalized TEC subset numbers and thymic architecture. Thymopoiesis and thymic output were supranormal, leading to the accelerated peripheral reconstitution of naïve CD4 and CD8 T-cells with a broad Vβ repertoire and decreased homeostatic T-cell proliferation. Combined therapy facilitated T:B cooperativity and enabled a B-cell humoral response to a CD4 T-cell dependent neoantigen challenge early post-BMT. In vivo antigen-specific CD8 T-cell responses and clearance of a live pathogen was superior with combined versus individual agent therapy. Thus, KGF combined with androgen blockade represent a novel approach to restore thymic function and facilitates the rapid recovery of peripheral T-cell function following allogeneic BMT.
INTRODUCTION

Allogeneic bone marrow transplantation (BMT) is a valuable treatment option for malignant and nonmalignant disorders. Following myeloablative conditioning, a favorable outcome depends upon successful immune reconstitution, including the de novo generation of a polyclonal population of naïve T-cells in the thymus. Mature T-cell generation is substantially delayed post-BMT, primarily due to thymic injury induced by pre-BMT chemoradiotherapy and graft-versus-host disease (GvHD). Fungal and viral infections normally controlled by T-cells can occur at high frequency in BMT patients, resulting in significant morbidity and mortality. Thus, strategies are needed to speed thymopoiesis post-BMT.

Normal thymopoiesis involves a program of thymocyte differentiation and maturation through sequential stages characterized by CD4 and CD8 expression—CD4-CD8- (“double negative”, DN), CD4+CD8+ (“double positive,” DP), and CD4+ or CD8+ (“single positive,” SP)—culminating in the export of mature CD4+ and CD8+ T-cells into the periphery. The thymic stroma is composed primarily of a three-dimensional matrix of cortical and medullary thymic epithelial cells (TEC). TEC directly support thymocyte development and selection but are susceptible to BMT-conditioning-induced damage, impairing the ability of the thymus to produce T-cells for prolonged periods of time after BMT.

Several growth factors regulate the development, proliferation and function of TEC throughout life, including Fibroblast Growth Factor-7 (FGF-7), also known as keratinocyte growth factor (KGF). KGF is an epithelial growth factor mainly produced by mesenchymal cells in the thymus and binds exclusively to a specific
member of the fibroblast growth factor receptor-2 family, FGFR2-IIIb (KGFR), which is expressed in the thymus by TEC\textsuperscript{17}. KGF can aid in the protection and/or repair of epithelial cells in murine models of radiation- and chemotherapy-induced injury and is FDA-approved for the prevention of oral mucositis associated with chemoradiotherapy and BMT\textsuperscript{18-20}. Murine studies have demonstrated that thymic injury and prolonged immune deficiency can be prevented by KGF pretreatment in models with and without GvHD\textsuperscript{11,21,22}. KGF also has been shown to facilitate engraftment and abrogate GvHD-induced lethality in murine BMT recipients\textsuperscript{23}.

The thymic atrophy that occurs with advancing age has been partly linked to physiological changes in sex steroid hormone production\textsuperscript{24-26}. Androgen receptors (AR) are expressed on TEC, certain thymocyte subsets and mature T-cells although the exact mechanisms by which androgens exert their effects on thymopoiesis and T-cell homeostasis/function are not fully understood\textsuperscript{27-31}. Physical castration of aged mice results in a complete restoration of thymic size and function to pre-pubertal levels and mice castrated pre-BMT restore thymopoiesis and peripheral T-cell numbers more rapidly than sham-castrated recipients\textsuperscript{32-36}. While physical castration has been proven effective in the murine model, methods of chemically induced castration are more directly translatable to the human BMT setting. Disrupting sex steroid production using a LHRH agonist (LHRH-A) rapidly results in long-lasting changes in sex steroids similar to that of surgical castration\textsuperscript{37}. Leuprolide acetate (Lupron) is a potent LHRH-A that is currently used in the clinic to treat prostate cancer and LHRH-A has been tested as a single agent in a pilot study of autologous and allogeneic HSCT recipients and shown to increase levels of naïve CD4\textsuperscript{+} T-cells in the periphery in a cohort of patients\textsuperscript{38}.
The receptor distribution of FGFR2-IIIb and ARs on TEC indicates the potential for additive effects from combined treatment with KGF and Lupron. We hypothesized that pre-BMT androgen blockade via chemical castration could act in an additive fashion with KGF to enhance thymic recovery and T-cell reconstitution in allogeneic BMT recipients. This study focuses on two currently FDA-approved agents (rhuKGF, Kepivance™ and leuprolide acetate, Lupron™). We report that combined pre-BMT treatment resulted in a restoration of thymic architecture, number and subset distribution of TEC. These changes led not only to additive effects on restoring thymopoiesis, thymic output and recovery of peripheral naïve CD4 and CD8 T-cell numbers but also in vivo responses to neoantigen and challenges with a live pathogen. These findings suggest a novel, clinically translatable approach to accelerate the recovery of functional immune system recovery following BMT.

MATERIALS AND METHODS

Animals

C57BL/6 (H-2^b^; termed B6) and [C57BL/6xBalb/c]F_1 (H-2^{b}d^{b}; termed CB6F1) male mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and used at 8 weeks of age as BMT recipients or controls (nonBMT controls). Donor female BALB/c (H-2^d^) or C57BL/6.Ly5.1 mice of the same age were purchased from the National Cancer Institute (Frederick, MD). Mice were housed in specific pathogen-free facilities. All protocols were approved by IACUC at UMN.
KGF Administration and Chemical Castration

rhuKGF (kindly provided by Amgen, Thousand Oaks, CA) was administered subcutaneously for 3 consecutive days (5 mg/kg per day) prior to radiation treatment as previously reported\(^\text{11}\). Leuprolide acetate (Lupron, TAP Pharmaceuticals, Lake Forest, Illinois), a LHRH agonist that ablates sex steroid hormone production, was injected as a 3-month deposition into the hind leg of B6 recipients 13 days prior to TBI at a dose of 0.8 mg/mouse based upon dose response studies in the same model demonstrating equivalency in day 28 post-BMT thymopoiesis in doses of 0.4, 0.8 and 1.2 mg/mouse\(^\text{34}\) (and data not shown).

BM Transplantation

Single cell suspensions of BM cells obtained from femurs and tibiae of Balb/c (allogeneic) or B6.Ly5.1 (congenic) donors were CD4/8-depleted as described\(^\text{39}\) and \(10^7\) (allogeneic) or \(5 \times 10^6\) (congenic) CD4/8-depleted BM cells were intravenously administered to recipients that had received 11 Gy total body irradiation (TBI) from a cesium source 24h prior to BMT.

Lymphocyte Flow Cytometry

Thymocytes, splenocytes and lymph nodes were suspended in 2% FCS/PBS and \(10^6\) cells were incubated with appropriate fluorochrome-conjugated monoclonal antibodies (BD Pharmingen, San Jose, CA) for 30 minutes at 4°C. A total of \(10^4\)-\(10^5\) live events were acquired on a FACScalibur flow cytometer (BD Pharmingen, San Jose, CA) and analyzed with FlowJo software (TreeStar, San Jose, CA).
TEC analysis by FACS

TEC were isolated and analyzed as described\textsuperscript{40,41}. Individual thymi were removed and small capsule incisions were made, followed by gentle disruption in ice-cold RPMI to deplete majority of thymocytes. Thymi were then incubated twice for 15 min in 0.1% collagenase-D plus 0.125% DNase-I (Roche, Indianapolis, IN), followed by incubation for 30 min in 0.1% collagenase/dispose plus 0.125% DNase-I (Roche). Supernatants from the final two enzymatic digestions were pooled and analyzed with the following antibodies: CD45-PerCp, Ly51/CDR1-FITC, MHC-II-PE (BD Pharmingen), and biotinylated-\textit{Ulex-europaeus}-agglutinin-1 (UEA-1) (Vector Labs, Burlingame, CA) plus streptavidin-conjugated-Cy5 (Molecular Probes, Eugene OR). Anti-AIRE antibody was a generous gift of H. Scott (Monash University, Australia) and was detected with mouse anti-rat IgG2c-Cy5 (BD Pharmingen).

BrdU incorporation assays

Mice were injected intraperitoneally with 200\textmu l of a 5mg/ml solution of BrdU/PBS on days 3, 10 or 22 post-BMT, and subsequently given BrdU in their drinking water for 5 days (0.8 mg/ml). Recipients were sacrificed on indicated days and TEC or T-cells were assessed for BrdU incorporation using the BrdU Flow Kit (BD) and the Alexa-647-conjugated monoclonal anti-BrdU antibody (clone PRB-1, Invitrogen). At least $5 \times 10^4$ CD45$^+$ (for TEC analyses) or total live events (for lymphocyte analyses) were acquired by FACS.
Detection of recent thymic emigrants

Anesthetized mice were injected in one thymic lobe with 10µl of a 5mg/ml solution of sulfo-NHS-LC biotin in PBS (Pierce, Rockford IL). After 24h, thymus and spleen were stained with streptavidin-conjugated-Cy5 and other markers and analyzed by flow cytometry as described42.

Immunofluorescence microscopy

Intact thymi embedded in OCT (Sakura, Tokyo, Japan) were snap-frozen in liquid nitrogen and stored at ~80ºC. Cryosections (7µm) were fixed by air-drying overnight, blocked with 10% normal horse serum/PBS (Jackson Immunoresearch, West Grove, PA) and stained with rabbit-anti-mouse cytokeratin-5 (K5) antibody (Covance, Berkeley, CA) plus a cyochrome-5-conjugated goat-anti-rabbit antibody (Molecular Probes) and biotinylated mouse-anti-mouse K18 (Progen Biotechnik, Heidelberg, Germany) plus Alexa-555-conjugated streptavidin (Molecular Probes). Sections were mounted under a cover slip with DAPI anti-fade solution (Molecular Probes) and imaged on the following day at RT using an Olympus FluoView 500 Confocal Scanning Laser Microscope (Olympus, Center Valley, PA). Identification of TEC subsets according to marker expression was done as previously reported22.

Immunization with KLH and quantification of serum immunoglobulin

Mice were injected i.p. with 50µg KLH (CalBiochem, La Jolla, CA) in Complete Freund’s Adjuvant (Sigma) followed three weeks later with 50µg KLH in Incomplete Freund’s
Adjuvant (Sigma). After 7 days, peripheral blood was collected by retro-orbital bleeds and analyzed for total and KLH-specific IgG1 levels by ELISA\textsuperscript{43}.

**Listeria monocytogenes infection**

The recombinant *L. monocytogenes* strains *Lm*-OVA and $\Delta$actA-$Lm$-OVA\textsuperscript{44} (attenuated) expressing full-length chicken ovalbumin were kindly provided by Dr. S.S. Way (University of Minnesota). Mice were inoculated with early logarithmic phase ($O.D_{600}$ of 0.1) bacteria grown in brain heart infusion broth at 37° C. Mice were injected intravenously with $10^6$ CFU of $\Delta$actA-$Lm$-OVA (primary) or $10^5$ CFU of *Lm*-OVA (secondary) diluted in 200µl PBS.

**Quantification of Lm-OVA-specific CD8 T-cells**

MHC-I-DimerX:mouse-Ig-PE was purchased from BD and purified OVA\textsubscript{257-64} (SIINFEKL) peptide was purchased from Anaspec, San Jose, CA. MHC-I-DimerX:mouse-Ig:OVA\textsubscript{257-64} conjugates were prepared according to manufacturer's instructions (BD). Peripheral blood was incubated with DimerX:mouse-Ig:OVA\textsubscript{257-64}-PE plus antibodies for other markers (all from BD) and $5\times10^3$ donor CD8 T-cells were collected and analyzed by flow cytometry.

**Determination of *L. monocytogenes* CFU**

Mice were immunized intravenously with $10^6$ CFU and rechallenged with $2\times10^6$ CFU of *Lm* strain 2C\textsuperscript{45} diluted in 200µl PBS. Four days after secondary infection, livers were removed and homogenized in 0.05% Triton X-100/PBS (Sigma, St. Louis, MO). Serial
dilutions were plated onto BHI plates and *Lm* colonies were enumerated after 24-48h at 37ºC.

**Statistical Analysis**

Differences between treated and untreated BMT groups were analyzed by a two-tailed, paired Student’s t-test with unequal distribution.

**RESULTS**

**Combined pretreatment with KGF and Lupron additively restores thymopoiesis early after allogeneic BMT**

KGF administered prior to TBI and BMT enhanced thymopoiesis and peripheral T-cell reconstitution following BMT with maximal benefit observed in the periphery two months post-BMT\(^1\). To determine whether KGF and androgen blockade prior to BMT could act in an additive fashion to more rapidly restore thymopoiesis, allogeneic murine BMT recipients of rigorously T-cell-depleted BM cells were either left untreated (BMT Control) or pre-treated with KGF, Lupron or KGF+Lupron prior to transplant. On d28 post-BMT, total thymocyte cellularity was ~50% reduced in BMT controls compared with age/sex-matched, unmanipulated B6 controls (nonBMT Control) (Figure 1A). Mice treated with KGF or Lupron abrogated the reduction in thymocyte cellularity seen in BMT controls. Combined treatment with KGF+Lupron showed an additive increase in thymic cellularity to numbers that were significantly greater than untreated, KGF- or Lupron-treated BMT recipients and nonBMT controls (Figure 1A). The relative
distribution of thymocyte subsets was not affected by any treatment with KGF and/or Lupron; therefore, concomitant increases in the DN, DP, CD4+ SP and CD8+ SP subsets were observed (Figure 1A-E). Additive effects of KGF+Lupron were maintained through at least d56 post-BMT (Supplemental Figure S1A-E).

Since both donor-derived and residual host-derived thymocytes might contribute to thymic cellularity early post-BMT, we assessed thymocyte chimerism. On d28 post-BMT, >95% of thymocytes were of donor origin, increasing to >99% by d56 (data not shown). Therefore, increased thymic cellularity was due to increased numbers of donor-derived thymocytes, not preservation of host-derived thymocytes.

**Combined pretreatment with KGF and Lupron maximally restores TEC post-BMT**

To determine whether protection/restoration of TEC was responsible for the enhancement of thymopoietic recovery observed on d28 post-BMT, absolute numbers of TEC, defined as CD45 MHC-II+ cells, were quantified in thymi of allogeneic BMT recipients left untreated or pre-treated with KGF, Lupron or KGF+Lupron and compared to nonBMT controls. Regardless of pre-treatment, TEC were severely depleted as early as d7 post-BMT (>75%) and TEC numbers remained significantly reduced through d14 post-BMT when compared with nonBMT controls (data not shown). On d26 post-BMT, all BMT groups had still reduced total TEC numbers compared with nonBMT controls except for KGF+Lupron-treated BMT recipients that had restored TEC to levels above untreated BMT recipients and to similar numbers compared with nonBMT controls (Figure 2A). With the exception of Lupron-treated BMT recipients, cortical TEC (cTEC; CD45+MHC-II+Ly51+) were restored to normal levels at this time point (Figure 2B).
Medullary TEC (mTEC; CD45-MHC-II<sup>+</sup>Ly51<sup>-</sup>) remained depleted in all groups except for the KGF+Lupron BMT recipients that had similar mTEC numbers compared to nonBMT controls (Figure 2C).

Within the medulla, two distinct mTEC subpopulations can be identified based upon MHC-II expression and binding to the lectin *Ulex Europaeus Agglutinin-1* (UEA-1)-mTEC<sub>lo</sub> (CD45-MHC-II<sub>lo</sub>Ly51<sup>-</sup>UEA-1<sup>+</sup>) and mTEC<sub>hi</sub> (CD45-MHC-II<sub>hi</sub>Ly51<sup>-</sup>UEA-1<sup>+</sup>)<sup>40,41</sup>. Both mTEC populations were reduced in number by ≥ 50% in untreated, KGF- or Lupron-treated BMT recipients compared with nonBMT controls on d26 post-BMT (Figure 2D-E). However, KGF+Lupron treatment resulted in comparable mTEC<sub>lo</sub> and mTEC<sub>hi</sub> numbers as nonBMT controls and 1.5-fold (mTEC<sub>lo</sub>) and 4-fold (mTEC<sub>hi</sub>) higher than untreated BMT controls (Figure 2D-E). Numbers of AIRE<sup>+</sup> mTEC<sub>hi</sub>, known to be critical for the negative selection of thymocytes with autoreactive specificities<sup>46</sup>, were significantly reduced (~3-fold) in untreated and Lupron-treated BMT recipients compared with nonBMT controls. AIRE<sup>+</sup> mTEC<sub>hi</sub> numbers were restored in KGF- and KGF+Lupron-treated BMT recipients to levels similar to nonBMT controls (Figure 2F).

Medullary dendritic cells in the thymus, also involved in negative selection<sup>46</sup>, were severely depleted following BMT and were significantly and maximally restored by d26 post-BMT in KGF+Lupron-treated BMT recipients (data not shown).

To determine whether the increased numbers of TEC observed on d26 post-BMT were due to enhanced TEC proliferation at an earlier time point, mice were injected between d10-14 post-BMT with the thymidine analogue BrdU, which is incorporated into DNA of replicating cells. In agreement with the relative TEC numbers observed on d26
post-BMT, total TEC, cTEC and mTEC\textsuperscript{hi} showed the greatest levels of proliferation in the thymi of BMT recipients treated with KGF+Lupron (Figure 2G-J).

In addition to the loss of TEC, immunofluorescence microscopy showed a loss of clear compartmentalization of cortex and medulla in the thymi of untreated BMT recipients on d28 post-BMT (Supplemental Figure S2). In contrast, nonBMT controls displayed well organized and densely clustered cortical and medullary regions with a distinct cortico-medullary boundary, a finding largely recapitulated in KGF+Lupron-treated BMT recipients (Supplemental Figure S2). All groups had restored thymic architecture by d56 post-BMT (Supplemental Figure S2).

**Pretreatment with KGF and Lupron results in enhanced T-cell reconstitution in the lymph nodes and spleen by d35 post-BMT.**

To determine whether improved thymopoiesis induced by KGF and Lupron resulted in higher numbers of peripheral T-cells, lymph nodes of BMT recipients were analyzed for CD4\textsuperscript{+} and CD8\textsuperscript{+} T-cells on d35 post-BMT. CD4\textsuperscript{+} and CD8\textsuperscript{+} T-cell numbers in untreated BMT recipients were reduced ~50% and ~75%, respectively, compared with nonBMT controls, and were partially restored in BMT recipients pre-treated with KGF or Lupron alone (Figure 3A,C). Combined, these treatments provided an additive increase in CD4\textsuperscript{+} and CD8\textsuperscript{+} T-cells that was significantly greater than either agent alone (Figure 3A,C), completely eliminating CD4\textsuperscript{+} and virtually eliminating CD8\textsuperscript{+} T-cell lymphopenia. Because small numbers of radioresistant host-derived peripheral T-cells still remained at this time point, we specifically quantified naïve, donor-derived, CD4\textsuperscript{+} and CD8\textsuperscript{+} T-cells to assess the contribution of thymus-derived T-cells to reconstitution.
of peripheral T-cells. Additive increases of donor-derived, naïve CD4+ (H2Kd+CD4+CD45RBhiCD44lo) and CD8+ (H2Kd+CD8+CD62LhiCD44lo) T-cells were observed in BMT recipients treated with KGF+Lupron that were significantly greater than was observed in untreated, KGF- and Lupron-treated BMT recipients, respectively (Figure 3B,D). By day 60 post-BMT, untreated BMT recipients had restored their CD4+ T-cell compartment to nonBMT control levels while Lupron- and KGF+Lupron treated BMT recipients maintained comparatively higher numbers of total and naïve CD4+ T-cells (Supplemental Figure S3A,B). Donor-derived, naïve CD8+ T-cells remained significantly reduced through d60 post-BMT in all BMT groups (compared with nonBMT controls) except those pretreated with KGF+Lupron (Supplemental Figure S3C,D).

Similar to the lymph node findings, combined treatment with KGF+Lupron resulted in complete reconstitution of splenic T-cell numbers at d35 post-BMT. Whereas there were highly significant (>4-fold) reductions in total CD4 and CD8 T-cells in the spleen of untreated BMT recipients compared with nonBMT controls (Figure 3E,G), combined treatment completely prevented the state of T-cell lymphopenia, resulting in the highest increase in total CD4 and CD8 T-cells, which appeared additive for total CD8 T-cells but not for total CD4 T-cells (Figure 3E,G). Compared to BMT controls, naïve, donor-derived CD4 T-cells were significantly increased by KGF (3-fold), Lupron (4-fold) and KGF+Lupron (5-fold), reaching levels that were no longer significantly lower than nonBMT controls (Figure 3F). Significant increases in naïve, donor-derived CD8 T-cells were observed with KGF or Lupron (3-fold), and KGF+Lupron (5-fold) compared with untreated BMT recipients (Figure 3H). Total and naïve, donor-derived CD8 T-cell numbers remained deficient through d60 post-BMT and only the combined treatment
resulted in a significant restoration of these cells compared to untreated controls (Supplemental Figure S3G,H). These data demonstrate that combined treatment with KGF+Lupron prior to BMT additively and durably enhances recovery of donor-derived, naïve CD4+ and CD8+ T-cells in both lymph nodes and spleen post-BMT. Analysis of TCR Vβ repertoire by flow cytometric analysis representing 12 Vβ alleles confirmed that KGF and Lupron do not affect the diversity of TCR Vβ usage in donor-derived T-cells compared to non-BMT controls (Supplemental Figure S4).

**Combined pretreatment with KGF and Lupron result in enhanced thymic output and less homeostatic proliferation early post-BMT.**

Since KGF and Lupron each may increase peripheral T-cell expansion, we sought to determine the relative contributions of thymic export and peripheral homeostatic expansion to the observed increased numbers of total and naïve peripheral CD4 and CD8 T-cells found in secondary lymphoid organs. Thymic export was quantified at d28 post-BMT by detection of intrathymically biotin-labeled, donor-derived T-cells that had recently emigrated to the spleen42. Consistent with higher naïve splenic CD4 T-cell numbers, we observed a significant and almost significant increase in the export of donor-derived CD4 T-cells into the periphery in BMT recipients treated with KGF and Lupron, respectively, compared to the untreated BMT controls (Figure 4A). Although thymic export of donor-derived CD8 T-cells was not significantly improved with KGF or Lupron alone, there was a significant and additive increase in the export of donor-derived CD4 and CD8 T-cells into the periphery in KGF+Lupron treated BMT recipients on d28 post-BMT, consistent with higher naïve CD4 and CD8 T-cell numbers in this group (Figure 4A,B).
To correlate thymic output with the degree of homeostatic proliferation (HP) occurring in peripheral lymphoid organs, BrdU was given continuously in the drinking water between d30-35 post-BMT. BrdU incorporation with upregulation of CD44 in CD4 and CD8 T-cells in the spleen and lymph nodes was measured. An inverse relationship was observed between the number of recently exported CD4 and CD8 T-cells into the periphery around d28 post-BMT and the degree of HP detected in peripheral lymphoid organs between d30-35 post-BMT (Figure 4C-E). The higher donor-derived, naïve CD4 and CD8 T-cell numbers are likely due to increased thymic output rather than higher levels of HP.

**Pretreatment with KGF and Lupron enhances T-dependent antibody responses early post-BMT**

The prolonged CD4+ T-cell deficiency following BMT can preclude the generation of normal immune responses to T-cell-dependent B cell antigens. Since KGF+Lupron treated BMT recipients had normalization of donor naïve CD4 T-cells and donor B-cells by day 35 post-BMT (data not shown), we sought to determine whether this treatment could enhance a humoral immune response against a T-cell-dependent neoantigen challenge given on d28 post-BMT. Mice were immunized with KLH plus adjuvant, rechallenged two weeks later with KLH and serum immunoglobulin levels measured after seven days. Total and KLH-specific IgG1 levels were ~50% lower in untreated BMT recipients compared with nonBMT controls and pretreatment with KGF or Lupron only marginally improved T-cell dependent B-cell isotype switching as measured by anti-KLH specific IgG1 antibody levels (Figure 5A,B). BMT recipients treated with
KGF+Lupron produced significantly greater amounts of total and KLH-specific IgG1 antibody reaching levels comparable to nonBMT controls (Figure 5A,B). The early increases of peripheral donor T-cells and B-cells in BMT recipients pretreated with KGF+Lupron permits the normal generation of a CD4 T-cell dependent B-cell immunoglobulin isotype switched response to KLH even when given as early as d28 post-BMT.

**Pretreatment with KGF and Lupron enhances functional immune response against L. monocytogenes post-BMT**

To determine whether improved immune reconstitution induced by pre-BMT treatment with KGF and/or Lupron would permit a functional immune response to challenge with a live intracellular pathogen post-BMT, mice were immunized with $10^6$ CFU of an attenuated strain of *Listeria monocytogenes*. To monitor pathogen specific immune responses, a congenic BMT system was utilized with *Listeria monocytogenes* (*Lm*) engineered to express chicken ovalbumin ($\Delta$actA-Lm-OVA). Thus, activated, *Lm*-specific CD8 T-cell responses were quantifiable in peripheral blood by MHC class-I:OVA tetramer binding to donor-derived, CD44$^+$ CD8 T-cells (Ly5.1$^+$CD8$^+$CD44$^+$K$^b$:OVA$^{257-264}$). Seven days after primary infection, KGF- or Lupron-treated BMT recipients contained modestly higher numbers of donor-derived, OVA-specific CD44$^+$ CD8 T-cells compared with untreated BMT recipients and treatment with KGF+Lupron resulted in additively higher numbers that were significantly greater than all other BMT groups (Figure 6A). Post-mortem necroscopic analysis revealed that all
BMT and nonBMT groups had effectively cleared this attenuated strain by 3 weeks post-infection (data not shown).

To investigate the effects of KGF and Lupron on a secondary immune response against *Lm-OVA*, mice were rechallenged 42d after primary infection with $10^5$ CFU of the non-attenuated parent strain, *Lm-OVA*. Three days later, we restimulated splenocytes from infected animals ex vivo with soluble OVA$_{257-264}$ and found that only KGF+Lupron-treated BMT recipients demonstrated a comparable number of IFN$\gamma^+$ CD8 T-cells as nonBMT controls, $\geq$ 2-fold higher than all other groups (Figure 6B).

In separate experiments, untreated, KGF-, Lupron- or KGF+Lupron-treated allogeneic BMT recipients were assessed for clearance of *Lm* as measured by CFU determination in livers of infected animals four days after secondary infection. Untreated BMT recipients contained significantly higher bacterial burdens than nonBMT controls and KGF or Lupron administered as single agents prior to BMT resulted in a marginal benefit for decreasing liver CFU (Supplemental Figure S5). Notably, KGF+Lupron pretreated BMT recipients significantly reduced the CFU burden from the liver to levels that were comparable to nonBMT controls (Supplemental Figure S5).

**DISCUSSION**

We report a novel therapy given entirely prior to the BMT conditioning regimen that combines the administration of KGF and the LHRH-agonist (Lupron) to enhance thymopoiesis and peripheral T-cell recovery and function in BMT recipients. Compared to untreated BMT recipients, KGF+Lupron treatment additively increased thymocyte and
peripheral T-cell recovery. Thymic architecture and TEC were significantly restored in KGF+Lupron-treated recipients early post-BMT, improving peripheral donor-derived T-cell reconstitution due to increased thymic output. KGF+Lupron pre-treated BMT recipients mounted a superior immune response to the neoantigen, KLH, and cleared a live infection with Lm more effectively than other treatment groups.

In rodents and non-human primate models of chemoradiotherapy and BMT, KGF pretreatment has been shown to augment thymopoiesis in an IL-7-dependent fashion. Since TECs are KGFR+, the putative mechanism of action proposed was stimulation of TEC proliferation and/or repair from radiation-induced injury. In untransplanted mice, KGF administration increased TECs numbers. Thus, KGF may induce the expansion of a TEC progenitor population that, in turn, may rapidly mature to restore TEC numbers following BMT conditioning. Androgen blockade by physical castration can reverse the thymic atrophy accompanying aging and can improve thymopoietic recovery in rodents undergoing allogeneic BMT. While our experiments focused on male mice, gonadal hormone blockade should be effective in females since Lupron has been successfully used to treat hormone–related disorders in women. ARs are expressed on TEC, non-TEC thymic stromal cells, thymocytes and peripheral T-cells, although chimeric studies have indicated that the restorative effect of castration on the thymus required AR expression on TEC. Since castration-induced enhancement of post-BMT thymopoietic recovery was KGF-independent, these data suggest that each agent acts through distinct mechanisms.

Thymic cellularity is tightly controlled by the availability of thymic stromal niches to support thymocyte maturation. While studies have demonstrated TBI-induced TEC
depletion\textsuperscript{10-12}, this is the first to quantitatively analyze TEC subset depletion and recovery by FACS following TBI and BMT. TBI-induced TEC depletion is not surprising in light of data illustrating that TEC, especially mTEC, are dynamically proliferating in the steady state\textsuperscript{46}. The equivalent loss of TEC observed in all BMT groups early post-BMT (d7, d14) indicates that KGF+Lupron accelerates the restoration rather than the protection of TEC compartment from TBI-induced depletion. KGF and Lupron appeared to act in concert to restore more rapidly TEC numbers without disrupting thymic architecture and may have provided for a larger stromal scaffold to support thymopoiesis. These proliferation events may have occurred early post-BMT based upon earlier studies demonstrating maximal TEC proliferation at 1-3 days after discontinuation of KGF\textsuperscript{14}. KGF has been shown to be mitogenic for both cTEC and mTEC and can act to restore the thymic disorganization associated with aging and BMT (14; GAH, unpublished data). A large percentage of UEA-1\textsuperscript{+} mTEC express FGFR2-IIIb\textsuperscript{14}. While expression patterns of AR on TEC subsets have not been described, androgen removal is mitogenic for mTEC\textsuperscript{51}. It is thought that mTEC\textsuperscript{hi} encompass mature, post-mitotic TEC that are derived from immature mTEC\textsuperscript{lo} \textsuperscript{46}. Our findings of a large number of BrdU\textsuperscript{+} mTEC\textsuperscript{hi} in KGF+Lupron-treated BMT recipients are consistent with the interpretation that these cells are the progeny of mTEC\textsuperscript{lo} recovering from depletion and maturing to reconstitute mTEC\textsuperscript{hi} cells. Thus, KGF+Lupron appears to maximally enhance mTEC\textsuperscript{hi} regeneration post-BMT, including the AIRE\textsuperscript{+} subset, critical for the expression of tissue-restricted antigens needed for negative repertoire selection of autoreactive T-cell clones during thymopoiesis\textsuperscript{52}.
The additive increase in thymic cellularity in recipients pretreated with KGF+Lupron correlated with increased thymic export of T-cells into the periphery. Previous studies have shown that KGF treatment and androgen blockade (physical castration), when analyzed individually, could increase thymic cellularity and enhance thymic output\cite{14,35,36}. In untransplanted mice, thymic export is a direct function of thymic cellularity\cite{53}. Our data suggest that the same relationship holds true following BMT, even though the thymus is damaged and exports T-cells into a lymphopenic environment. In agreement with an enhanced thymic output in KGF+Lupron treated mice, we observed by d35 post-BMT additive increases in donor-derived, naïve CD4 and CD8 T-cells in both spleen and lymph node. Because T-cell-depleted BM was used, all donor-derived T-cells with a naïve surface phenotype must have been generated in the thymus in this transplant model. The BMT models employed in these studies do not result in GvHD clinically or histologically. Because androgen blockade by physical castration pre-BMT does not exacerbate GvHD in other models and KGF has been shown to ameliorate thymic damage and lethality due to GvHD\cite{22,23,36}, KGF+Lupron may represent a viable clinical approach to speeding immune reconstitution even in the setting of GvHD risk.

Higher export of naïve T-cells in KGF+Lupron-treated BMT recipients also correlated with a decrease in HP observed in secondary lymphoid organs of these mice. T-cells derived from lymphopenia-induced peripheral expansion or expansion driven by exogenous cytokines generally result in expansion of a limited number of peripheral T-cell clones and can create “holes” in the TCR repertoire\cite{5,54-56}. In our studies, TCR Vβ diversity was not skewed by KGF and/or Lupron. Therefore, KGF+Lupron in the clinical
BMT setting may speed reconstitution of a diverse naïve peripheral T-cell compartment capable of responding to a wider array of pathogens post-BMT.

Poor immune responsiveness among BMT recipients leads to suboptimal responses to immunization for extended periods of time\textsuperscript{5,47}. In myeloablated autologous hematopoietic graft recipients, KLH responses were decreased up to 16 months\textsuperscript{47}. Although all BMT groups mounted similar primary anti-KLH IgM responses (data not shown), indicating that B-cell function was sufficient, only KGF+Lupron provided maximal benefit for enhancing T:B cooperativity with higher total IgG1 and KLH-specific IgG1 antibody titers indicative of isotype switching mechanisms requiring CD4 T-cell help\textsuperscript{57}. The demonstration that pretreatment with KGF+Lupron improved the T-cell-dependent antibody response suggests a potential for KGF and Lupron as adjunct to immunization in BMT recipients.

Low peripheral CD4 and CD8 T-cells numbers results in poor immune responsiveness and increased susceptibility to and severity of infection among BMT recipients\textsuperscript{7,58}. Similarly, we observed a severe defect in the ability of untreated murine BMT recipients to clear \textit{Lm} from the liver, whereas KGF+Lupron pretreated BMT recipients cleared \textit{Lm} from these sites as efficiently as nonBMT controls. Consistent with this defective clearance, untreated BMT recipients showed $<50\%$ of \textit{Lm}-reactive CD8 T-cells compared with nonBMT controls and BMT recipients pre-treated with KGF+Lupron following a secondary infection. This same trend was observed when analyzing the percentage of IFN-$\gamma^+$ cells within the \textit{Kb}:OVA-reactive CD8 T-cell compartment (data not shown). Because adaptive immunity against many intracellular pathogens involve highly conserved T-cell responses\textsuperscript{59-61} the enhanced \textit{Lm} immunity
would likely be extended to a wider spectrum of viral and intracellular bacterial pathogens that pose a serious threat to BMT recipients. In this regard, we have shown that KGF-treated non-human primate BMT recipients develop improved responses to a challenge with simian immunodeficiency virus compared to untreated BMT recipients. T-cell responsiveness to CD3 signals also has been shown to be drastically diminished post-BMT compared to healthy controls and cytomegalovirus reactivation post-BMT has been associated with dysfunctional antigen-specific CD8 T-cells. In contrast, peripheral T-cells from castrated mice are hyper-responsive to antigen and CD3/28 signaling early after castration, although this effect lost by 7-weeks post-castration. While heightened sensitivity to antigenic stimulation of T-cells following androgen blockade may have played a role in driving early peripheral T-cell reconstitution, it is likely not a major contributor to improved CD8 T-cell responses to *Lm* late post-BMT.

In summary, combined KGF and androgen blockade was highly effective in speeding thymic recovery and peripheral T-cell reconstitution post-BMT. Treated BMT recipients were resistant to a pathogenic challenge and relatively early after engraftment were capable of T:B-cell cooperativity in generating antibody responses to a neoantigen. Since KGF and Lupron are being used as single agents in the context of human BMT, our data suggests future clinical trials designed to determine the safety and efficacy of combining these agents for facilitating immune recovery post-BMT.
Authorship

Contribution: R.M.K. designed and performed research, analyzed and interpreted data, made the figures and wrote the paper; S.L.H performed research; A.P-M. provided advice and edited the paper; P.A.T. performed research; R.L.B. provided advice and edited the paper; G.A.H. advised on experimental design and edited the paper; B.R.B. designed research, advised on experimental design, and edited the paper.

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Figure Legends

Figure 1. Pretreatment with KGF combined with androgen blockade additively restore thymopoiesis early after allogeneic BMT. Lethally irradiated B6 recipients of allogeneic (Balb/c) bone marrow were left untreated (BMT Control) or pretreated with KGF, Lupron or KGF+Lupron and analyzed for thymocyte cellularity at day 28 post-BMT alongside age/sex-matched, unmanipulated B6 controls (nonBMT Control). Data shown are mean absolute numbers ± SEM of (A) total thymocytes and of the thymocyte subsets: (B) CD8^−CD4^− double-negative, (C) CD8^−CD4^+ double-positive, (D) CD4^+CD8^− single-positive, and (E) CD4^+CD8^+ single-positive. The data are representative of 4 independent experiments with 4-5 mice per group; *, p<0.05 compared with untreated BMT recipients; #, p<0.05 compared with KGF-treated BMT recipients.

Figure 2. Combined pretreatment with KGF and androgen blockade maximally restores numbers of total TEC and mTEC subsets by day 26 post-BMT. (A-F) Lethally irradiated B6 recipients of allogeneic (Balb/c) bone marrow were left untreated (BMT Control) or pretreated with KGF, Lupron or KGF+Lupron and analyzed for absolute numbers of TEC and TEC subsets at day 26 post-BMT. Single cell suspensions were prepared from enzymatic digests of individual thymi and used to determine (A) total thymic epithelial cells (TEC; CD45^−MHC-II^+), (B) cortical TEC (cTEC; CD45^−MHC-II^+Ly51^+), (C) medullary TEC (mTEC; CD45^−MHC-II^+Ly51^−), (D) UEA-1^+ mTEC^lo (CD45^−MHC-II^+Ly51^− UEA-1^+), (E) UEA-1^+ mTEC^hi (CD45^−MHC-II^+Ly51^− UEA-1^+), (F) AIRE^+ mTEC^hi (CD45^−MHC-II^+Ly51^− AIRE^+). (G-J) Total TEC and individual TEC subsets were assessed for proliferation by continuous administration of BrdU in the
drinking water (0.8mg/ml) between day 10 and 14 post-BMT at which point (G) Total TEC, (H) cTEC, (I) mTEC\textsuperscript{lo} and (J) mTEC\textsuperscript{hi} subsets were analyzed for BrdU incorporation. Data shown are the mean numbers of TEC ± SEM or mean percentages of BrdU\textsuperscript{+} TEC ± SEM and are representative of one experiment of 4 mice per group; *, p<0.05 compared with BMT controls; #, p<0.05 compared with KGF-or Lupron-treated BMT recipients.

**Figure 3. Combined pretreatment with KGF and androgen blockade significantly restore numbers of total and donor-derived, naïve CD4\textsuperscript{+} and CD8\textsuperscript{+} T-cells in lymph node and spleen by day 35 post-BMT.** Lethally irradiated B6 recipients of allogeneic (Balb/c) bone marrow were left untreated (BMT Control) or pretreated with KGF, Lupron or KGF+Lupron and analyzed for the presence of T-cells in the lymph nodes and spleen at day 35 post-BMT alongside unmanipulated age/sex-matched B6 controls (nonBMT Control). Mean absolute numbers ± SEM of (A,E) total CD4\textsuperscript{+} T-cells, (B,F) naïve (CD45RB\textsuperscript{high}CD44\textsuperscript{low}) CD4\textsuperscript{+} T-cells, (C,G) total CD8\textsuperscript{+} T-cells, and (D,H) naïve (CD62L\textsuperscript{high}CD44\textsuperscript{low}) CD8\textsuperscript{+} T-cells in the lymph nodes and spleen are shown. Lymph node data is represented here by pooled cells from 2 inguinal, 2 axillary and mesenteric lymph nodes. Data are representative of 3 experiments, each with 4 mice per group; *, p<0.05 compared with BMT controls; #, p<0.05 compared with KGF-treated BMT recipients.

**Figure 4. Enhanced thymocyte cellularity in KGF+Lupron-treated BMT recipients correlates with increased T-cell export into the periphery and diminished
homeostatic proliferation early post-BMT. (A, B) Lethally irradiated B6 recipients of allogeneic (Balb/c) bone marrow that were left untreated (BMT Control) or pretreated with KGF, Lupron or KGF+Lupron were intrathymically injected (one thymic lobe) with biotin at 28 days post-BMT. After 24 hours of in vivo labeling, the export of thymus-derived (A) CD4 and (B) CD8 T-cells into the periphery was assessed by staining total splenocytes with fluorescently labeled streptavidin in combination with monoclonal antibodies specific for CD4 and CD8. Absolute numbers of recent thymic emigrants (RTE) were normalized to absolute numbers of donor-derived CD4+ or CD8+ T-cells in (A) and (B), respectively. (C-F) Homeostatic proliferation in the CD4+ and CD8+ T-cell compartment was also assessed between day 30-35 post-BMT. Treated and untreated BMT recipients and nonBMT controls were injected i.p. with BrdU (1mg) on day 30, followed by continuous administration of BrdU in the drinking water (0.8mg/ml) through d35 post-BMT at which point CD4+ and CD8+ T-cells were analyzed for BrdU incorporation and concomitant upregulation of CD44, thus indicating that proliferation had occurred within the 5-day labeling window. Data shown are the mean percentages of BrdU+CD44+ ± SEM of total CD4+ T-cells (C,E) and CD8+ T-cells (D,F) isolated from the spleens and lymph nodes of these mice. These data are representative of one experiment with 4 mice per group; *, p<0.05 compared with BMT controls; #, p<0.05 compared with KGF- or Lupron-treated BMT recipients.

Figure 5. KGF treatment and androgen blockade prior to BMT enhance the secondary humoral immune response to KLH after allogeneic BMT. Lethally irradiated C57BL/6 recipients of allogeneic (Balb/c) bone marrow were left untreated
(BMT Control) or pretreated with KGF, Lupron or KGF+Lupron and immunized at day 28 post-BMT with 50 μg keyhole limpet hemocyanin (KLH) in Complete Freund’s adjuvant (KLH/CFA) alongside unmanipulated age/sex-matched C57BL/6 controls (nonBMT Control). Two weeks after primary immunization (equivalent to day 42 post-BMT), mice were rechallenged with 50 μg KLH in Incomplete Freund’s adjuvant (KLH/IFA). Serum was then collected after 7 days and analyzed for (A) total IgG1 and (B) KLH-specific IgG1 antibody levels by ELISA. Data shown are mean μg IgG1 per mL serum ± SEM from one experiment with 4 mice per group; *, p<0.05 compared to untreated BMT controls.

Figure 6. Combined pretreatment with KGF and androgen blockade prior to BMT significantly improves CD8 T-cell responses against *Listeria monocytogenes* after allogeneic BMT. Lethally irradiated C57BL/6 Ly5.2+ recipients of congenic (C57BL/6 Ly5.1+) bone marrow were left untreated (BMT Control) or pretreated with KGF, Lupron or KGF+Lupron and immunized at day 42 post-BMT alongside unmanipulated age/sex-matched B6 controls (nonBMT Control). For primary immunization, 10^6 CFU of an attenuated strain of *L. monocytogenes* that express recombinant full-length chicken ovalbumin (∆actA-Lm-OVA) was intravenously injected. (A) Absolute numbers of donor-derived Ly5.1+CD44+CD8+ Kb-OVA257-64-specific T-cells were quantified in peripheral blood of infected animals by FACS 7 days after primary infection. (B) Immunized mice were then rechallenged with 10^5 CFU of the virulent parent strain, Lm-OVA, 42 days after primary infection. After 3 days, isolated splenocytes of infected animals were restimulated ex vivo for 5h with OVA_{257-64} and
donor-derived CD8 T-cells were analyzed for IFNγ production by FACS. *, p<0.05 compared with untreated BMT controls; #, p<0.05 compared with KGF- and Lupron-treated BMT recipients.
FIGURES

Figure 1

Thymus cellularity at day 28 post-BMT

A. Total

B. CD4CD8\(^-\) (DN)

C. CD4\(^+\)CD8\(^+\) (DP)

D. CD4\(^+\)CD8\(^-\) SP

E. CD4\(^+\)CD8\(^+\) SP

Legend:
- BMT Control
- KGF
- Lupron
- KGF+Lupron
- nonBMT Control
Figure 2

Thymic epithelial cellularity at day 26 post-BMT

A. Total TEC
B. Cortical TEC
C. Medullary TEC

D. UEA-1<sup>-</sup> mTEC<sup>lo</sup>
E. UEA-1<sup>-</sup> mTEC<sup>hi</sup>
F. AIRE<sup>+</sup> mTEC<sup>hi</sup>

Thymic epithelial cell turnover between day 10-14 post-BMT

G. Total TEC turnover
H. cTEC turnover
I. mTEC<sup>lo</sup> turnover
J. mTEC<sup>hi</sup> turnover
Figure 3

Lymph node cellularity at day 35 post-BMT

A. Total CD4+ T cells
B. Naive CD45RB+CD44lo CD4+ T cells
C. Total CD8+ T cells
D. Naive CD62L+CD44lo CD8+ T cells

Spleen cellularity at day 35 post-BMT

E. Total CD4+ T cells
F. Naive CD45RB+CD44lo CD4+ T cells
G. Total CD8+ T cells
H. Naive CD62L+CD44lo CD8+ T cells
Figure 4

A. Donor CD4⁺ RTE in spleen

B. Donor CD8⁺ RTE in spleen

C. CD4⁺ T cells undergoing HP in spleen

D. CD8⁺ T cells undergoing HP in spleen

E. CD4⁺ T cells undergoing HP in LN

F. CD8⁺ T cells undergoing HP in LN
Figure 5

(A) Total IgG1

(B) KLH-specific IgG1
Figure 6

A. Primary response

- BMT
- $10^6$ CFU atten-Lm
- PB FACS

Responding K$^b$:OVA-reactive donor CD8 T cells in peripheral blood

- BMT Control
- KGF
- Lupron
- KGF+Lupron
- nonBMT Control

B. Secondary response

- BMT
- $10^6$ CFU atten-Lm
- $10^5$ CFU Lm
- restim splenocytes w/ OVA

IFN$^\gamma$+ donor CD8 T cells in spleen

$10^6$ IFN$^\gamma$+ donor CD8 T cells per spleen
Keratinocyte growth factor and androgen blockade work in concert to protect against conditioning regimen-induced thymic epithelial damage and enhance T-cell reconstitution following murine bone marrow transplantation

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