THYMUS

Production of BMP4 by endothelial cells is crucial for endogenous thymic regeneration

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The thymus is not only extremely sensitive to damage but also has a remarkable ability to repair itself. However, the mechanisms underlying this endogenous regeneration remain poorly understood, and this capacity diminishes considerably with age. We show that thymic endothelial cells (ECs) comprise a critical pathway of regeneration via their production of bone morphogenetic protein 4 (BMP4). ECs increased their production of BMP4 after thymic damage, and abrogating BMP4 signaling or production by either pharmacologic or genetic inhibition impaired thymic repair. EC-derived BMP4 acted on thymic epithelial cells (TECs) to increase their expression of Foxn1, a key transcription factor involved in TEC development, maintenance, and regeneration, and its downstream targets such as DLL4, a key mediator of thymocyte development and regeneration. These studies demonstrate the importance of the BMP4 pathway in endogenous tissue regeneration and offer a potential clinical approach to enhance T cell immunity.

INTRODUCTION

Continuous generation of adaptive immune diversity is dependent on T cell development in the thymus. T cell development is a complex process involving the continuous differentiation and development of thymocytes in close interaction with, and under the instruction of, the surrounding stromal microenvironment, which is composed of endothelial cells (ECs), fibroblasts, and highly specialized thymic epithelial cells (TECs). Signals stemming from the microenvironment that drive T cell development include the Notch ligand Delta-like 4 (DLL4), cytokines such as interleukin-7 (IL-7) and stem cell factor, and chemokines such as CXCL12, CCL19, CCL21, and CCL25 (1).

Notwithstanding its importance for generating and maintaining an important arm of adaptive immunity, the thymus is not only extremely sensitive to damage, which can come in the form of stress (corticosteroids), cytokreduction chemotherapy, infection, sex hormones, surgery, and irradiation (2) but it also has a remarkable capacity for repair. The general phenomenon of endogenous thymic regeneration has been known for even longer than its immunological function (3, 4); however, the underlying mechanisms controlling this process remain unstudied. Although there is likely continual thymic involution and regeneration in response to stress and infection in otherwise healthy people, acute and profound thymic damage, such as that caused by common cancer cytoreductive therapies or the conditioning regimes as part of hematopoietic cell transplantation (HCT), leads to prolonged T cell deficiency, precipitating high morbidity and mortality from opportunistic infections and may even facilitate cancer relapse (5, 6). Furthermore, the continuous decline in thymic function with age markedly erodes the regenerative capacity of the thymus (2, 7). Therefore, there is a clinical need for therapeutic strategies to mediate rapid regeneration of thymic function after acute immune damage.

One approach to developing novel regenerative strategies is to understand and exploit the pathways underlying endogenous thymic regeneration. We have recently described one such pathway centered on the production of IL-22 by innate lymphoid cells (ILCs) (8). IL-22 is significantly up-regulated after damage, and mice deficient for IL-22 had a defect in their thymic regenerative capacity (8). However, although Il22−/− animals lagged behind wild-type controls in their ability to repair thymus function, there was still considerable regeneration in IL-22−/− mice (8), suggesting that other pathways likely play a role during endogenous thymic regeneration.

RESULTS

BMP signaling pathways are up-regulated after thymic damage

To identify alternate regeneration pathways in the thymus, we performed an unbiased transcriptome analysis of the nonhematopoietic (CD45−) stromal cell compartment of the thymus, which is less sensitive to thymic damage compared with the CD45+ hematopoietic compartment (fig. S1A). To detect gene changes likely important for regeneration, we focused on days 4 and 7 after a sublethal dose of total damage.
body irradiation (TBI), critical time points that immediately precede observable cellular regeneration (day 4) and when significant but incomplete regeneration is under way (day 7; fig. S1B). Using this approach, we found significant up-regulation at both days 4 and 7 of several genes known to be involved in thymic function, including Foxn1, Dil4, Kitl, Cxcl12, Il7, and Fgf7 (Fig. 1A), many of which have been described to promote thymic regeneration when given exogenously or activated genetically (2). However, in addition to these canonical thymopoietic factors, we also found significant up-regulation of Bmp4, which is a critical factor during the development of multiple organs, including the thymus, where it can target both stromal and hematopoietic compartments (9). Reflecting this increase in Bmp4 expression, we could also identify a significant enrichment at both days 4 and 7 after TBI in genes downstream of bone morphogenetic protein receptor (BMPR) signaling [Gene Ontology (GO) number: 0030510; Fig. 1B]. These gene changes were confirmed at the protein level by a significant increase in the intrathymic levels of BMP4 from days 7 to 14 after TBI (Fig. 1C). However, although the absolute levels of BMP4 do not increase until day 7, reflecting the increase in BMP signaling observed before the increase in absolute BMP4 (Fig. 1, B and C), we found a significant increase in the relative amounts of BMP4, suggesting an increase in the bioavailability of BMP4 as early as day 2 (Fig. 1D). Consistent with a localized effect, mice that received targeted irradiation to the mediastinum (which locally targets the region encompassing the thymus) have also increased the availability of BMP4 (fig. S1C). Together, these findings suggest that BMP signaling pathways are activated during the regenerative response in the thymus after damage.

### BMP4 induces TECs to up-regulate Foxn1 and its downstream targets after damage

The cognate receptor for BMP4 is a heterodimer made up of two subunits: a nonredundant type II receptor, BMPR2, and one of the two type I receptors, BMPR1A or BMPR1B, which signal through Smad1/5/8 (10). Analysis of the cellular distribution of these receptor subunits revealed widespread expression in the thymus, although non-hematopoietic stromal cells expressed Bmpr1a two to three logs higher than thymocytes (fig. S2). Although there was detectable expression of Bmpr1a and Bmpr2 by all TEC subsets, higher expression of the nonredundant Bmpr2 subunit was detected on cortical TECs (cTECs) compared with medullary TECs (mTECs; Fig. 2A). BMP4 signals can also contribute to the differentiation of pluripotent stem cells toward the TEC lineage (11, 12), possibly via its ability to directly induce up-regulation of Foxn1 (13), a forkhead box transcription factor that is not only critical for TEC development and maintenance (14, 15) but can also confer TEC identity on cells such as fibroblasts (16). Consistent with the differential expression of the Bmpr2 by TECs, Foxn1 expression was significantly increased at days 4 and 7 after TBI in purified cTECs but not in mTECs (Fig. 2B). Although the nonredundant function for Foxn1 in the thymus has been known for decades (14, 17), its role in regeneration is only beginning to be understood (18, 19). Consistent with a role for Foxn1 during endogenous thymic regeneration, significant changes were found at days 4 and 7 after TBI in the expression of a large proportion of the FOXN1 targets identified by the Boehm and Holländer groups (20–22). Specifically, 66 and 68% of FOXN1 targets were significantly changed at days 4 and 7, respectively, and 79% were significantly changed at either day 4 or 7 after TBI (Fig. 2, C and D, and table S1). Subsequent gene set enrichment analysis (GSEA) confirmed these findings showing a significant enrichment in these downstream FOXN1 targets at both days 4 and 7 after thymic damage (Fig. 2E). Although there was a significant increase in Foxn1 expression between days 4 and 7 in cTECs (Fig. 2B), we did not observe a considerable change in FOXN1 target gene expression between days 4 and 7 after TBI (Fig. 2, C to E).

### BMP4 produced by ECs is a critical mediator of endogenous thymic regeneration

Consistent with our hypothesis that BMP signaling is important during endogenous thymic regeneration, mice treated with the pan BMP inhibitor dorsomorphin dihydrochloride (12.5 mg/kg) beginning 1 day before TBI exhibited significantly worse thymic recovery compared with phosphate-buffered saline (PBS)–treated controls (Fig. 3A), including all thymocyte and stromal populations analyzed (fig. S3, A and B). To confirm these pharmacologic findings, we generated iGremelin::K5-CreER+ (iGrem+TEC) mice, in which the expression of the secreted BMP inhibitor Gremlin is induced by CreER driven by

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**Fig. 1.** BMP signaling pathways are up-regulated in the thymus after thymic damage. (A and B) Thymuses were pooled from 6-week-old C57BL/6 mice were pooled, and microarray analysis was performed on CD45+ cells enriched from either untreated mice (d0) or 4 and 7 days (d4 and d7, respectively) after TBI (550 cGy; n = 3 per time point with each n pooled from three to five mice). (A) Volcano plot outlining genes that changed >1.5-fold, P < 0.05 with some key thymus-related genes highlighted. (B) GSEA was performed on the transcriptome derived from CD45+ cells after TBI (Fig. 1A) with BMP target genes (GO: 0030510). NES, normalized enrichment score. (C and D) Thymuses were harvested at days 0, 2, 4, 7, 10, 14, and 21 after TBI (n = 5 to 14 per time point), and BMP4 levels were measured by ELISA. (C) Absolute amount of BMP4 in the thymus. (D) Amount of BMP4 normalized to the weight of the thymus (ng BMP4/µg thymus). Data combined from two to three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001. ns, not significant.
the keratin 5 promoter leading to tamoxifen-induced expression of Gremlin in mTECs. Upon tamoxifen administration, these mice exhibited significantly worse thymic regeneration after TBI compared with IGremlin:K5-CreER (IGremlin) control mice (Fig. 3B and fig. S3, C and D).

To determine the cellular source of BMP4 in the thymus, we FACS (fluorescence-activated cell sorting)–purified cell populations comprising 99.5% of the thymus [including all thymocyte subsets, TECs, dendritic cells (DCs), ECs, and fibroblasts] at steady state and measured the expression of Bmp4 by quantitative polymerase chain reaction (qPCR). This analysis revealed that the only cell subsets within the thymus to produce detectable levels of Bmp4 mRNA were fibroblasts and ECs (Fig. 3C). However, when we FACS-purified these two populations at days 0 and 4, ECs but not fibroblasts exhibited an increased expression of Bmp4 (Fig. 3D). Pharmacologic inhibition of BMP signaling using dorsomorphin dihydrochloride inhibits multiple BMP signaling pathways, whereas induction of Gremlin inhibits signaling from BMP-2, BMP-4, and BMP-7. To conclusively determine the role of EC-derived BMP4 during thymic regeneration, we crossed BMP4h8 mice with Cdh5(PAC)-CreERT2 mice (BMP4h8/EC), enabling tamoxifen-induced deletion of BMP4 specifically in ECs (fig. S4, A and B), which express Cdh5 [vascular endothelial–cadherin (VE-cadherin)] (23). Although there was no immediate loss in thymic function when BMP4 was deleted at baseline (fig. S4C), if BMP4 was deleted immediately before TBI, then there was a significant loss of thymic recovery compared with controls (Fig. 3E and fig. S4D).

**ECs represent a damage-resistant niche within the thymus**

Our previous data revealed that ILCs, which can contribute toward endogenous thymic regeneration, were extremely damage-resistant (8). We analyzed the depletion of most cell subsets within the thymus 7 days after exposure to TBI and found that along with ILCs, ECs were resistant to acute thymic injury (Fig. 4A). This finding is consistent with a previous study that demonstrated that thymic ECs are resistant to damage (24). To confirm this finding, we analyzed ECs after damage caused by cytoreductive chemotherapy, corticosteroids, or TBI at a time point that is at the nadir of cellularity (fig. S5A) and results in extensive depletion of all subsets of thymocytes and TECs (fig. S5, B to D). In each of these models, we found that the number of ECs in the thymus remained remarkably unchanged (Fig. 4B), resulting in an increase in the proportion of these cells as the cellularity of the thymus decreased (Fig. 4C). Using TBI as a model of thymic damage (fig. S1B), we found that there was no change over time in the absolute number of ECs measured by flow cytometry (Fig. 4D). Furthermore, given the depletion of cells in the thymus, the proportion of ECs increased as a proportion of CD45− nonhematopoietic stromal cells (Fig. 4E) and total thymus cellularity (Fig. 4F). However, although there was no change in the absolute number of ECs, visualization using light-sheet fluorescence...
microscopy (LSFM) on the whole thymus after TBI revealed that the volume of the vasculature decreased commensurate to the rest of the thymus after damage (Fig. 4, G and H, and movies S1 to S4). Reflecting this change in vascular volume, there was a significant decrease in the total number of vessel segments at days 4 and 7 after TBI, but this had largely returned to baseline levels by day 14 (Fig. 4I). Similarly, the length of each vessel segment was reduced significantly after TBI, leading to a profound loss in total vessel length, but had begun regenerating by day 14 after TBI (Fig. 4I). Similarly, vessel branching, which is a critical vascular function, declines precipitously immediately after damage, slowly regenerating by day 14 (Fig. 4, K to L). However, although there are profound changes to vascular architecture after damage, there was a significant increase in the density of the vasculature after TBI (Fig. 4M).

Administration of ex vivo–propagated ECs promotes thymic regeneration in a BMP4-dependent fashion

Although the role of ECs during tissue regeneration has traditionally been attributed to their role in vascularization, including in the thymus (25), recent works have suggested that ECs can also contribute toward regeneration via their production of so-called angiocrine factors (26–28). Using a technique to constitutively activate the Akt pathway in ECs using the prosurvival adenosine 3′,5′-cyclic nucleotide phosphodiesterase 1F (EaORF1) (29), ECs can be propagated and expanded ex vivo (exEC) while maintaining their phenotype and vascular tube formation capacity (fig. S6, A and B). Consistent with this, GSEA comparing transcriptomes derived from freshly isolated TECs or exEC(Thymus) revealed no significant enrichment for gene signatures related to EC differentiation, branching, angiogenesis, or EC apoptosis (fig. S6C), although, perhaps unsurprisingly given its constitutive activation of Akt (30), there was enrichment for genes associated with EC proliferation and mild enrichment for EC migration (fig. S6C). Intravenous administration of 1 × 10^6 exECs derived from the thymus [exEC(Thymus)] 72 hours after TBI (Fig. 5A) led to significantly increased thymic cellularity compared with control mice on day 9 after irradiation (Fig. 5B). Although unchanged in their proportion (Fig. 5, C and D), this increased cellularity was reflected within both TEC and thymocyte subsets (Fig. 5E and fig. S6D), driven, at least in part, by increased cTEC proliferation (Fig. 5F). These in vivo data support our in vitro studies, which showed that BMP4 could directly promote proliferation of C9 but not TE-71 TEC cell lines (representing cTECs and mTECs, respectively; Fig. 5G). Together, these data are consistent with the differential BMP4 receptor expression we had observed in TECs (Fig. 2A). Intriguingly, although considerable benefit to thymic regeneration was observed with exEC(Thymus), we observed no effect on thymus regeneration when exECs were derived from cardiac or kidney ECs [exEC(Heart) or exEC(Kidney); Fig. 5, A to E], consistent with the previously demonstrated heterogeneity between ECs from different tissues and the tissue specificity of EC-derived regeneration (31).

Using this model of exogenous administration, the total number of ECs in the thymus was also significantly increased in mice that received exEC(Thymus) (Fig. 5H). Unlike TECs, we did not observe any change in EC proliferation after exEC(Thymus) administration (Fig. 5I). Together, this suggests that exECs derived from the thymus are preferentially able to enter the thymus to mediate regeneration. However, to conclusively track the ability of exECs to enter the thymus, we transplanted 10 × 10^6 carboxyfluorescein diacetate succinimidyl ester–positive (CFSE+) exECs 72 hours after TBI. Using this approach, we could detect a small population of CFSE+ ECs in the thymus at 4 hours after transfer (Fig. 5J). Supporting the notion that exEC(Thymus) do get in to the thymus, we observed the same capacity for thymic regeneration when 100 times fewer cells were injected directly into the thymus compared with when cells were injected intravenously (fig. S6E). Moreover, this regenerative benefit of exEC administration was persistent because we could still detect increased cellularity within the thymus for at least 28 days after TBI (Fig. 5K).

Administration of exEC(Thymus) induced up-regulation of Foxn1 by TECs (Fig. 6A), but reflecting the differential expression of Bmpr2 (Fig. 2A), this increase was only observed in cTECs and not in mTECs. Highlighting this mechanism of regeneration, we also found significant up-regulation of the FOXN1 target genes Dll4, Kitl, and Cxcl12 (Fig. 6B), all of which are critical for steady-state T cell development and reconstitution (20, 32–34). Although our studies suggest that the effects of BMP4 are primarily restricted to cTECs, surprisingly, conditioned medium (CM) from exEC(Thymus) induced Smad1/8 phosphorylation in both the C9 and TE-71 cell lines (Fig. 6, C and D), indicating that either BMP4 can signal through mTECs but that its effects are independent of Foxn1 and proliferation or that additional factors in the CM could be inducing Smad1/8 phosphorylation.

Although the expression of Foxn1 decreases considerably when TECs are immortalized, including the C9 cTEC cell line (fig. S7A), its expression can be induced in C9 cells by BMP4 (fig. S7B). Supporting the hypothesis that BMP4 production by exECs leads to thymic regeneration via induction of FOXN1, incubation of C9 cells for 24 hours with the CM from exEC(Thymus) was enough to induce the expression of Foxn1.
Fig. 4. ECs form a damage-resistant regenerative niche in the thymus. (A) Cell subsets in the thymus were assessed at day 7 after TBI, and the depletion was calculated compared with an untreated age-matched control cohort (n = 10 to 25 per subset). Subsets analyzed include DN1 (CD44+CD25−), early T-lineage progenitor (ETP; CD44+CD25−c-kit+), DN2 (CD44+CD25−), DN3 (CD44+CD25−), DN4 (CD44+CD25−), DP, SP4, SP8, CD8+ or CD8− DCs, MHCIIhi or MHCIIlo cTEC hi/lo (CD45−EpCAM+MHCII+Ly51hi/lo), fibroblast, ECs, and ILCs (CD45+CD3−CD8−IL7Rα+IL25+). (B and C) Six-week-old female C57BL/6 mice were treated with PBS (n = 10), Dex (50 mg/kg, ip, on day 0; n = 10), or TBI (550 cGy on day 0; n = 10). On day 4, mice were perfused with 25 μg of anti–VE-cadherin antibody (BV13) conjugated to Alexa Fluor 647 and sacrificed, and total thymic cellularity and EC number were assessed. (B) Total thymic cellularity and absolute number of ECs. (C) Concatenated flow cytometry plots detailing the proportion of VE-cadherin+CD45− cells in the thymus. SL-TBI, sublethal TBI. (D) Total cellularity (open circles) and absolute number of ECs (closed circles) in the thymus. Total thymus and vasculature. (I) Total number of vessel segments in the thymus vasculature after damage where segments were defined as the length of the vessel between two branching points. (J) Vessel segments were binned according to their length. Total vasculature length was calculated. (K) Vascular segments were color-coded on the basis of branch level. (L) Number of segments per branch level and the total number of vessel branches. (M) Vascular density was calculated as a ratio of vascular network volume and as a function of total thymus volume [from (H)]. Bar graphs represent means ± SEM. *** P < 0.001, ** P < 0.01, * P < 0.05; ns, not significant.
of Foxn1 in a similar fashion to recombinant BMP4 (Fig. 6E), an effect that could be abrogated with the addition of the BMP inhibitor Noggin (Fig. 6F). Similar to our in vivo results, we found that the CM from exEC(Thymus) led to up-regulation of both Dll4 and Kitl in C9 cells (Fig. 6G). exECs derived from the thymus expressed significantly more BMP4 (transcript and protein) compared with exECs derived from the heart or kidney (Fig. 6, G and H) and were subsequently able to induce Foxn1 in C9 cells, whereas exECs from the kidney or heart were unable to induce Foxn1 (Fig. 6I). Together, these data offer an explanation for the differential thymic regenerative capacities of these populations.

Last, to test the impact of BMP4 on exEC-mediated thymic regeneration, we generated exEC(Thymus) with BMP4 silenced by short hairpin RNA (shRNA). As confirmed by qPCR, Bmp4 expression was reduced by about 90% in thymic exECs transduced with the shRNA compared with the scrambled control (Fig. 7C). Unlike the CM from scrambled shRNA (shScram), which induced Foxn1 expression when incubated with C9 cells, the CM from cultures of Bmp4-shRNA (shBmp4) exECs failed to up-regulate Foxn1 (Fig. 6I) or its downstream targets Dll4 and Kitl (Fig. 6K). In mice given TBI and treated with scrambled or shBmp4 exEC(Thymus) on day 3, we found that the increase observed when exEC(Thymus) were administered was abrogated if BMP4 was silenced in these cells (Fig. 6L).

**DISCUSSION**

Endogenous thymic regeneration is a critical process that allows for the renewal of immune competence after such everyday insults as stress and acute infection. However, prolonged thymic deficiency caused by age-related involution, repeated rounds of cytoreductive chemotherapy or chronic infection, is a significant clinical challenge that can lead to increased opportunistic infections, reduced response to vaccines, and decreased capacity for immune surveillance (2). Although there is a well-described role for BMP4 during thymus organogenesis (9, 35, 36) and BMP4 is a crucial mediator in the induction TEC-like cells from pluripotent stem cells (11, 12, 37), its postnatal role has not been well defined. Our findings demonstrate that ECs are a necessary postnatal source of BMP4 that drives thymic regeneration after injury, primarily via up-regulation of the key TEC transcription factor FOXP1 and its downstream targets. Our findings support previous in vitro data linking BMP4 signaling and the expression of Foxn1 (13, 38).

FOXP1 is a forkhead box transcription factor expressed during thymic ontogeny that is crucial for functionally enabling TECs to support T cell development (14, 39, 40). However, the role of FOXP1 is not only restricted to the formation of thymus but is also critical for ongoing TEC maintenance, and its declining expression likely contributes to age-related thymic involution (15, 41–44). Furthermore, there is increasing evidence that FOXP1 is also important during thymic regeneration, because forced induction of FOXP1 is capable of reversing age-related thymic atrophy (18, 19) and recombinant FOXP1 protein can enhance T cell reconstitution after HCT (45). However, despite its importance for thymic function, it was only recently in a series of elegant studies by Holländer and colleagues (21) that a comprehensive list of 450 high-confidence direct targets of FOXP1 were identified in cTECs. This list of targets verified the
Fig. 6. exEC-produced BMP4 mediates thymic regeneration via activation of Foxn1 in TECs. (A and B) Six- to 8-week-old C57BL/6 mice were given TBI, and 1 x 10^6 exECs were administered intravenously at day 3 after TBI. Thymus was harvested 4 days later, cTECs and mTECs were FACS-purified, and the expression of Foxn1 (A) and its downstream target genes Dil4, Kitl, and Cxcl12 in cTECs (B) was measured by qPCR (n = 8 to 9 per group). (C and D) The CM from in vitro cultures of exECs derived from the thymus was incubated with C9 or TE-71 cells for 24 hours (n = 7 to 10). Recombinant BMP4 (30 ng/ml) and/or Noggin (100 ng/ml) were added to the marked wells as controls. (E) Foxn1 measured by qPCR. (F) Expression of Dil4 and Kitl measured by qPCR. (G) BMP4 protein was measured by ELISA in exEC CM derived from the thymus, heart, or kidney (n = 4 per group). (I) The CM from exECs generated from the heart, kidney, or thymus was incubated with C9 cells for 24 hours after which the expression of Foxn1 was measured by qPCR. (J and K) The CM from exECs derived from the heart, kidney, or thymus was incubated with C9 cells for 24 hours after which the expression of Foxn1 (J) and/or the Foxn1 downstream genes Dil4 and Kitl (K) was assayed by qPCR. (L) Transduced exECs were transplanted into mice previously given TBI on day 3. Total thymus cellularity at day 9 after shBmp4 or shScram was measured by flow cytometry. (n = 10 per group). Bar graphs represent means ± SEM of at least two independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

four putative FOXN1 targets (Dil4, Kitl, Cxcl25, and Cxcl12) that had been previously identified (20, 42). Consistent with the hypothesis that FOXN1 is a crucial regulator of endogenous regeneration, our studies demonstrated that Foxn1 expression was increased in cTECs after damage but that there was also a significant enrichment in the expression of these direct FOXN1 target genes. Regulation of the Notch ligand DLL4 is particularly relevant for regeneration, not only because it is critically important for steady-state thymic function with DLL4 deletion leading to complete abrogation of T cell development (33, 34), but also because we have previously shown that the concentration of intrathymic DLL4 expression can profoundly affect T cell development and thymus size (46).

Several recent studies have found that ECs, rather than merely passive conduits, can have an active role in tissue repair via the production of so-called angiocrine factors (28). Evidence for this regenerative role of ECs has been found in the liver (mediated by Cxcr7, hepatocyte growth factor, and Wnt2) (27, 47), lung (mediated by matrix metalloproteinase 14) (26), and bone marrow hematopoiesis (mediated by Notch signaling) (48–50). In addition to providing further evidence of the endogenous tissue regeneration capacity of ECs, our studies in the thymus also propose a potential therapeutic strategy for promoting endogenous thymic regeneration using ex vivo–propagated Akt-activated exECs (30). Reflecting the findings in the lung and bone marrow (26, 48, 51), exEC administration could significantly promote thymic regeneration after acute injury but only when the transferred exECs were derived from the thymus and not from other tissues such as the heart or kidney. These findings further highlight the high degree of EC tissue specificity, particularly in their response to damage and role during regeneration (31). Further to the lymphostromal interactions that regulate T cell development and TEC function (I), there is a commensurate influence of TECs on EC function such that TEC-specific deletion of vascular endothelial growth factor A (VEGF-A), or insertion of a hypomorphic FOXN1 allele, leads to disruption of the thymic vasculature (52, 53). Thus, although it is clear that ECs can promote TEC function and regeneration after damage, it will be interesting to study the role that TECs play in guiding EC function, including their tissue specificity.

In both endogenous and exogenous regeneration models, our studies suggest that the effects of BMP4 are restricted mostly to cTECs but not mTECs. Specifically, we found that (i) in the endogenous setting
of regeneration after TBI, there is an increase in Foxn1 expression in purified cTECs but not in mTECs; (ii) recombinant BMP4 induces the expression of Foxn1 and proliferation in the C9 (cTECs) but not the TE-71 (mTECs) cell line; and (iii) exEC administration induces the expression of Foxn1 and leads to the proliferation of cTECs but not of mTECs. These findings can be explained by enriched expression of the nonredundant Bmpr2 receptor subunit on cTECs compared with mTECs. Given the identification of bipotent TEC progenitors (TEPCs) during development and in the postnatal thymus that share common phenotypic cTEC markers (54–58), together with the capacity of BMP4 to drive TEC differentiation from pluripotent progenitors and stimulate postnatal TEPCs (11, 12, 37, 59), it is possible that BMP4 stimulation of TEPCs drives thymic regeneration. Although there is evidence that at least one of these progenitor cells is not capable of robust postnatal maintenance and regenerative capacity (60), the relative contribution of these phenotypically distinct TEPCs toward endogenous thymic regeneration is currently unclear and will require further study. Furthermore, at least one mechanism by which BMP4 can aid in thymic repair (namely, increased the expression of Foxn1 and its downstream targets such as Dil4) does not necessarily depend on direct stimulation of TEPCs. However, although our data strongly suggests that BMP4 signaling promotes thymic regeneration primarily by stimulating cTEC function given that (i) BMP4 can regulate hematopoietic stem cell (HSC) function (61), (ii) Akt-activated ECs can promote HSC function in vitro and in vivo (48, 50, 62), and (iii) the input of hematopoietic progenitors from the bone marrow is critical to maintain thymic function and a limited supply of hematopoietic progenitors hinders T cell reconstitution after HCT (63), we cannot exclude the possibility that exECs have both direct and indirect effects in aiding thymic regeneration. Moreover, because we can only detect exECs transiently after transfer, it is not entirely clear whether they mediate their effect locally or extrathyMICALLY. Nevertheless, the requirement for exEC regeneration on BMP4 and our observance of similar downstream targets to those observed during BMP4-mediated endogenous regeneration strongly suggest an equivalent mechanism of regeneration. Regardless of their local or systemic mechanisms, exECs or BMP4 represents a potent therapeutic strategy to improve thymic regeneration.

Furthermore, although BMP4 appears to be critical for promoting TEC regeneration after injury given that BMP4 receptors are also expressed on some thymocyte populations and BMP4 has been reported to inhibit the differentiation of T cells (64–67), the observation that BMP4 expression by ECs peaks early after damage and returns to baseline levels in both absolute and relative amounts by day 21 is likely significant for coordinating this balance of functions.

Together, these studies not only identify a mechanism governing endogenous thymic regeneration but also offer potential therapeutic strategies for immune regeneration in patients whose thymus has been irrecoverably damaged. These strategies are novel candidates for therapies to enhance thymic regeneration and improving immune competence in patients whose thymic function has been compromised because of cytotoxic conditioning, infection, or age.

MATERIALS AND METHODS

Study design

The goal of this study was to identify and understand the mechanisms of endogenous thymic regeneration. The Institutional Animal Care and Use Committees at the Memorial Sloan Kettering Cancer Center (MSKCC), the Fred Hutchinson Cancer Research Center (FHCRC), and the University of Georgia approved these studies. Power calculations from past studies were used to calculate the number of mice needed to ensure statistical power. All animal studies were conducted on 3 to 25 biological replicates. No randomization or blinding was performed, but all results were confirmed by two or more independent experiments.

Mice

Inbred female C57BL/6 mice were obtained from the Jackson Laboratory. BMP4<sup>TEC</sup> mice were generated by crossing B6;129S4-Bmp4<sup>tm1[Vam]</sup>/J mice (obtained from the Jackson Laboratory) with Cdhs5(PAC)-CreERT2 (which was provided by R. Adams from the Max Planck Institute under a material transfer agreement through Cancer Research UK) (23). To induce deletion, tamoxifen (Sigma-Aldrich) was dissolved in corn oil and intraperitoneally administered at a dose of 40 mg/kg per day for 5 days starting 2 days before TBI. iGrem<sup>TEC</sup> mice were generated by N. Manley (University of Georgia) by crossing K5-CreER<sup>+</sup> mice with B6;129S1-Gt(Rosa)26Sor<sup>tm1[Grem]Svok</sup> mice (which were provided by S. Vokes at the University of Texas, Austin) (68). To induce the expression of Gremlin, tamoxifen was dissolved in corn oil and intraperitoneally administered at a dose of 40 mg/kg per day for 3 days starting 1 day before TBI.

To induce thymic damage, mice were given either sublethal TBI, cyclophosphamide (Cyco), or dexamethasone (Dex). TBI was given at a dose of 550 centigray (cGy) from a cesium source mouse irradiator with no hematopoietic rescue, Cyco was administered intraperitoneally in two doses of 100 mg/kg over 2 days, and Dex was administered intraperitoneally at a dose of 50 mg/kg. The BMP type I receptor inhibitor dorsomorphin dihydrochloride was given to the indicated mice at a dose of 12.5 mg/kg 1 day before TBI and then twice daily from day 1. Mice were maintained at the MSKCC (New York, NY), FHCRC (Seattle, WA), or at the University of Georgia (Athens, GA). Animals were allowed to acclimatize for at least 2 days before experimentation, which was performed according to the Institutional Animal Care and Use Committee guidelines.

Reagents

For the detection of BMP4, whole thymus lysates were prepared by homogenizing the tissue in radioimmunoprecipitation assay buffer [50 mM tris (pH 7.6), 150 mM NaCl, 1% NP-40, 1% SDS, 0.01% sodium deoxycholate, 0.5 mM EDTA, and protease inhibitors (Thermo Fisher Scientific, A32955)]. The resulting supernatant was quantified using the BMP4 enzyme-linked immunosorbent assay (ELISA) kit (LS-F13543, LSBio) and read on a Spark 10M plate reader (Tecan). For flow cytometry and cell sorting, surface antibodies against CD45 (30-F11), CD31 (390 or MEC13.3), VE-cadherin (BV13), TER-119 (TER-119), CD4 (RM4-5 or GK1.5), CD8 (53-6.7), T cell receptor <span class="math-container" math-display="true"><sup>β</sup></span> (H57-597), CD8 (53-6.7), CD4 (RM4-5 or GK1.5), CD8 (53-6.7), T cell receptor <span class="math-container" math-display="true"><sup>β</sup></span> (H57-597), CD3 (145-2C11), c<sup>Δ</sup> (2B8), CD25 (PC61), CD44 (IM7), IA/IE (MHC II; MS/114.15.2), epithelial cell adhesion molecule (EpCAM; G8.8), Ly51 (6C3), CD11c (HL3), L-7Rtr (A7R34), Nk4p46 (29A1.4), retinoic acid–related orphan receptor gamma 2 (RORγt; B2D), CCR6 (140706), and platelet-derived growth factor receptor α (PDGFRα; APAS) were purchased from BD Biosciences, BioLegend, or ebioscience. Ulex europaeus agglutinin 1 (UEA1), conjugated to fluorescein isothiocyanate or biotin, was purchased from Vector Laboratories. Flow cytometry analysis was performed on an LSR II (BD Biosciences), and cells were sorted on an Aria II (BD Biosciences) using FACSDiva (BD Biosciences) or FlowJo (TreeStar Software).
Individual or pooled single-cell suspensions of freshly dissected thymuses were obtained and either mechanically suspended or enzymatically digested as previously described (8, 46). CD45− cells were enriched by magnetic bead separation using an autoMACS (Miltenyi Biotec). To isolate or analyze TECs, mice were anesthetized with isoflurane, administered intravenously with BV13 antibody (anti-VE-cadherin; 250 μg/ml), and euthanized 15 min after injection.

**Generation of exECs**

exECs were generated as previously described (29). Briefly, CD45− VE-cadherin− cells were FACs-purified and incubated with lentivirus containing the E4ORF1 construct for 48 hours. Cell culture medium containing murine recombinant VEGF (10 ng/ml) and fibroblast growth factor 2 (20 ng/ml), endothelial growth supplement (bovine hypothalamus; Alfa Aesar), sb431542 (Tocris Bioscience), heparin (50 μg/ml; Sigma-Aldrich), 1% GlutaMAX (Life Technologies), 1% nonessential amino acids (Life Technologies), 1% Hepes buffer (Life Technologies), and 1% antibiotic-antimycotic (Life Technologies) was replaced every 48 hours.

**Coculture experiments**

Culture medium that had been conditioned with exECs for 48 hours was incubated with C9 cells (provided by A. Farr from the University of Washington) for 40 hours. Recombinant murine BMP4 (30 ng/ml) and recombinant murine Noggin (100 ng/ml) were purchased from PeproTech. In vitro cell proliferation was measured using the CellTitre 96 Non-Radioactive Cell Proliferation Assay (Promega).

**Microarray**

Thymic nonhematopoietic stromal cells were isolated using CD45 MACS cell depletion. Microarray analysis was performed on an Affymetrix MOE 430 A 2.0 platform in triplicate for untreated mice and on days 4 and 7 after TBI. To obtain sufficient RNA for every time point, TECs of several mice were pooled. All samples underwent a quality control on a Bioanalyzer to exclude the degradation of RNA. RNA extraction, control of RNA integrity with a bioanalyzer, and complementary RNA labeling and hybridization were performed by the Integrated Genomics Core Facility of the MSKCC. GSEA was performed using the GSEA tool v2.0 of the Broad Institute (http://software.broadinstitute.org/gsea). Comparisons were made to known signaling pathways from the GO database (GO numbers: 0045446, 0010594, 0001938, 0001763, 0002040, 2003351, and 0030510) and the list of published downstream FOXN1 targets (21). The microarray data used in this study have been deposited in the Gene Expression Omnibus (GEO) under accession number GSE106982.

**Polymerase chain reaction**

Reverse transcription PCR was performed with QuantiTect Reverse Transcription Kit (Qiagen). PCR was done on ABI 7500 (Applied Biosystems) or StepOnePlus (Applied Biosystems) with TaqMan Universal PCR Master Mix (Applied Biosystems). Relative amounts of mRNA were calculated by the comparative ΔCt method. TaqMan gene expression assays for qPCR, including Bmp4 (Mm00432087_m1), Foxn1 (Mm00433948_m1), Bmpr1a (Mm00477650_m1), Bmpr1b (Mm0032971_m1), Dll4 (Mm00444619_m1), Kitl (Mm00442972_m1), and Cxcl12 (Mm00445553_m1) were all purchased from Life Technologies.

**Short hairpin RNA**

To silence BMP4, exEC (Thymus) were incubated in polybrene (8 μg/ml) with a lentiviral construct containing Bmp4 shRNA or scrambled sequences encoding for target-specific 19- to 25-nucleotide shRNA with a 6–base pair loop (sc-39745-V, Santa Cruz Biotechnology). After 5 days, puromycin (2 μg/ml) was added to select for transduced cells.

**Microscopy**

LSFM was performed as previously described (60). Briefly, paraformaldehyde-fixed organs were dehydrated with increasing concentrations of ethanol and cleared with benzyl benzoate and benzyl alcohol at a 2:1 ratio. The organs were imaged by a custom-built laser-scanned LSFM at 5× magnification (numerical aperture, 0.15) and stitched using XvStitch 1.80 software. Volumes were calculated using Imaris software (Bitplane) by segmenting an autofluorescence channel and the VE-cadherin signal. Subsequent filament analysis using the module MeasurementPro yielded the vessel characterization measures where segments were defined as the length of the vessel separating two branching points. Data were exported for analysis with MATLAB (MathWorks).

**Statistics**

Statistical analysis between two groups was performed with the non-parametric, unpaired Mann–Whitney U test. Statistical comparison between three or more groups was performed with the non-parametric, unpaired Kruskal–Wallis test. In Fig. 4 (J and L), statistics were generated using a two-way analysis of variance (ANOVA) with Tukey’s multiple comparison test. All statistics were calculated using GraphPad Prism, and display graphs were generated in GraphPad Prism or R.

**SUPPLEMENTARY MATERIALS**

immunology.sciencemag.org/cgi/content/full/3/19/eaal2736/DC1

Fig. S1. Thymic response to damage.

Fig. S2. Distribution of BMPR subunits on thymic cell populations.

Fig. S3. Inhibition of BMP signaling abrogates endogenous thymic regeneration.

Fig. S4. Deletion of BMP4 in ECs abrogates endogenous thymic regeneration.

Fig. S5. Damage response in the thymus to corticosteroids, chemotherapy, and TBI.

Fig. S6. exECs can be propagated ex vivo and maintain an EC phenotype.

Fig. S7. Validating methods of inducing Foxn1 and silencing Bmp4.

Table S1. High-confidence FOXN1 target gene changes after TBI.

Movie S1. Thymic vascular architecture and branching by LSFM (day 0).

Movie S2. Thymic vascular architecture and branching by LSFM (day 4).

Movie S3. Thymic vascular architecture and branching by LSFM (day 7).

Movie S4. Thymic vascular architecture and branching by LSFM (day 14).

**REFERENCES AND NOTES**


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Competing interests: J.A.D., T.W., and M.R.M.v.d.B. are inventors on a patent application (US2015/058095) submitted by MSKCC that covers the use of Ecs and/or BMP4 for thymic regeneration. S.R. is a founder of (and unpaid consultant to) and D.N. and M.G. are employees of Angiocrine Bioscience, which holds patents on the platform technology surrounding E4ORF1 expression in Ecs. All other authors declare that they have no competing interests.

Data and materials availability: The microarray data used in this study have been deposited in the GEO under accession number GSE106982.
Regeneration circuits in the thymus

Chemotherapy and radiation treatments in cancer patients damage a number of tissues and organs, including the thymus. Prolonged thymic damage can lead to T cell deficiency and increase susceptibility to the development of opportunistic infections and malignancies. Here, Wertheimer et al. have examined thymic regeneration in mice after sublethal total body radiation and document a critical role for bone morphogenetic protein 4 (BMP4) signaling in thymic regeneration. They found endothelial cells to be a critical source of BMP4 and propose that BMP4 produced by endothelial cells induces the expression of the transcription factor FOXN1 in thymic epithelial cells to promote thymic regeneration. These studies should eventually facilitate the development of treatment regimens to promote immune competence in patients undergoing chemotherapy and radiation treatments.