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SHORT COMMUNICATION

Improving thymus implantation for congenital athymia with interleukin-7

Hyunjung Min[1,a], Laura A Valente[1,2,a], Li Xu[1], Shane M O'Neil[1], Lauren R Begg[1], Joanne Kurtzberg[1,3] & Anthony J Filiano[1,2,4,5]

[1]Marcus Center for Cellular Cures, Duke University, Durham, NC, USA
[2]Department of Pathology, Duke University, Durham, NC, USA
[3]Department of Pediatrics, Duke University, Durham, NC, USA
[4]Department of Neurosurgery, Duke University, Durham, NC, USA
[5]Department of Immunology, Duke University, Durham, NC, USA

Correspondence
AJ Filiano, Marcus Center for Cellular Cures, Duke University, Durham, NC, USA.
E-mail: anthony.filiano@duke.edu

[a]Equal contributors.

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Abstract

Objectives. Thymus implantation is a recently FDA-approved therapy for congenital athymia. Patients receiving thymus implantation develop a functional but incomplete T cell compartment. Our objective was to develop a mouse model to study clinical thymus implantation in congenital athymia and to optimise implantation procedures to maximise T cell education and expansion of naïve T cells. **Methods.** Using *Foxn1*^{nu} athymic mice as recipients, we tested MHC-matched and -mismatched donor thymi that were implanted as fresh tissue or cultured to remove donor T cells. We first implanted thymus under the kidney capsule and then optimised intramuscular implantation. Using competitive adoptive transfer assays, we investigated whether the failure of newly developed T cells to expand into a complete T cell compartment was because of intrinsic deficits or whether there were deficits in engaging MHC molecules in the periphery. Finally, we tested whether recombinant IL-7 would promote the expansion of host naïve T cells educated by the implanted thymus. **Results.** We determined that thymus implants in *Foxn1*^{nu} athymic mice mimic many aspects of clinical thymus implants in patients with congenital athymia. When we implanted cultured, MHC-mismatched donor thymus into *Foxn1*^{nu} athymic mice, mice developed a limited T cell compartment with notably underdeveloped naïve populations and overrepresented memory-like T cells. Newly generated T cells were predominantly educated by MHC molecules expressed by the donor thymus, thus potentially undergoing another round of selection once in the peripheral circulation. Using competitive adoptive transfer assays, we compared expansion rates of T cells educated on donor thymus *versus* T cells educated during typical thymopoiesis in MHC-matched and -mismatched environments. Once in the circulation, regardless of the MHC haplotypes, T cells educated on a donor thymus underwent abnormal expansion with initially more robust proliferation coupled with greater cell death, resembling IL-7 independent spontaneous expansion. Treating implanted mice with recombinant interleukin

(IL-7) promoted homeostatic expansion that improved T cell development, expanded the T cell receptor repertoire, and normalised the naïve T cell compartment. **Conclusion.** We conclude that implanting cultured thymus into the muscle of *Foxn1*^{nu} athymic mice is an appropriate system to study thymus implantation for congenital athymia and immunodeficiencies. T cells are educated by the donor thymus, yet naïve T cells have deficits in expansion. IL-7 greatly improves T cell development after thymus implantation and may offer a novel strategy to improve outcomes of clinical thymus implantation.

Keywords: primary immunodeficiency disorders, T cells, thymus, translational immunology, transplant immunology

INTRODUCTION

Congenital athymia is a rare disease associated with the failure to develop a functional thymus. This causes severe immunodeficiency because of the inability to generate functional T cells.[1] Without treatment, congenital athymia is usually fatal by 2 years of age as a result of infections.[2] Thymus implantation is therapy for congenital athymia using major histocompatibility complex (MHC)-unmatched thymus tissue discarded from infants undergoing elective surgeries to treat congenital heart disease.[3] In the USA, the therapy is FDA-approved (RETHYMIC) and in the UK offered by the GOSH Thymus Transplantation programme as tissue transplantation regulated by the Human Tissue Authority.[4] As of 2023, more than 150 total patients have been implanted since 1991, with an overall survival rate of 75%.[4–8] Patients with congenital athymia begin to reconstitute T cells from endogenous host stem and progenitor cells within 1 year after MHC-unmatched thymus implants.[9,10] Newly developed T cells can proliferate in response to *in vitro* antigenic stimulation (PHA, concavalin A and others), patients can clear new and pre-existing infections and can safely receive vaccinations.[9–11] This suggests that recipient T cells can interact and expand on recipient antigen-presenting cells (APCs) in the periphery.

While thymus implantation is beneficial for patients with complete athymia, the absolute number of total and naïve T cells fail to fully develop and peak at about 5–10% of a normal T cell compartment.[5,11] Additionally, although patients with complete athymia have underlying autoimmune co-morbidities, autoimmune complications post-implant are common and likely arise from underdeveloped central tolerance.[12] Understanding why newly developed T cells post-implant do not fully expand to a normal compartment and how this affects central tolerance is critical to optimise thymus implantation.

Thymus implantation into a postnatal, T cell-deficient host represents a unique scenario compared to typical foetal T cell development. There are differences in thymus structure, the transcriptional landscape of T cell progenitors entering the thymus, and obvious differences in the environment as T cells egress from the thymus.[13] Regardless of the scenario, once released from the thymus, newly matured T cells are maintained in the periphery through engagement of cognate self-antigens and cytokine support.[14,15] In a lymphopenic host, T cells undergo homeostatic or spontaneous expansion to fill the T cell compartment. Importantly, interleukin-7 (IL-7) is critical for homeostatic proliferation which is a slower process than spontaneous proliferation and creates a greater naïve T cell pool using cognate self-antigens.[16,17]

In this study, we developed a mouse model of thymus implantation using *Foxn1*^{nu} athymic recipient mice to further investigate and understand how the new recipient T cell pool is selected in an MHC-mismatched donor thymus implantation. Furthermore, we investigated the role of IL-7 in driving homeostatic expansion of newly developed T cells after thymus implantation with the goal of increasing proper naïve T cells and expanding the T cell compartment to normal levels.

RESULTS

Modelling clinical thymus implants using cultured neonatal thymus

To test T cell reconstitution after thymus transplants, we utilised *Foxn1*^{nu} nude mice. These mice have a spontaneous deletion mutation in the *Foxn1* gene

that is a critical transcription factor for the development of thymic epithelial cells. This prevents normal thymus development and causes severe immunodeficiency.[18] We harvested thymus from adult donor CD45.1$^+$ mice and immediately transplanted the tissue underneath the CD45.2$^+$ *Foxn1*^{nu} nude recipient mouse's kidney capsule. We refer to these as 'fresh transplants' indicating there was no manipulation of the thymus other than slicing into a smaller volume prior to intrarenal transplantation (Figure 1a). Donor-derived T cells (CD45.1$^+$) and recipient-derived T cells (CD45.2$^+$) can be tracked separately by the appropriate CD45 haplotype (Figure 1b, Supplementary figure 1a). We transplanted thymus into MHC-matched (congenic) or MHC-mismatched (allogeneic; to model clinical transplant conditions) mice. In fresh MHC-matched transplanted recipient mice, donor T cells from the thymus remained circulating in the blood for at least 15 weeks after transplant (Figure 1b; 66.1% of circulating T cells). However, in MHC-mismatched thymus recipients, donor T cells were mostly cleared by 15 weeks post-transplant (Figure 1b; 1.87% circulating T cells). In both MHC-matched (data not shown) and MHC-mismatched transplants (Figure 1c, Supplementary figure 1c), recipient-derived T cells (CD45.2$^+$) were detected as early as 5 weeks post-transplant and plateau at about 3% of total CD45.2$^+$ cells by 10 weeks (Figure 1c). In both conditions, T cells are skewed heavily to central memory (CD62L^{hi} CD44^{hi}) and effector memory (CD62L^{lo} CD44^{hi}) phenotypes with limited populations of naïve (CD62L^{hi} CD44^{lo}) T cells (Figure 1d, Supplementary figure 1b, d).

In human thymic implantation, donor thymus tissue is cultured for 2–3 weeks prior to implantation into patients with congenital athymia. It is thought that this process is critical in 'washing out' donor T cells from the thymic tissue, which could cause graft-*versus*-host disease in the immunocompromised recipient.[9] We aimed to model the clinical transplant protocol by using neonatal CD45.1$^+$ mice (aged 2–5 days) as thymus donors and culturing thymus for 1–2 weeks before implanting slices of the cultured thymus under the kidney capsules of CD45.2$^+$ *Foxn1*^{nu} MHC-mismatched recipients (Figure 1e, Supplementary figure 2a). T cells accumulated in the culture media and appeared to be depleted from the thymus by day 7 or 8 in culture while the percentage of thymic epithelial cells was enriched relative to a naïve thymus (Figure 1f and g, Supplementary figure 2b–d). Based on these data, we implanted neonatal thymus after a minimum of 8 days in culture. *Foxn1*^{nu} mice that were implanted with an MHC-mismatched cultured thymus produce recipient T cells by 5 weeks post-implantation and maintain low numbers of recipient T cells (average 2.3% CD3$^+$ of CD45.2$^+$) at 15 weeks post-implantation (Figure 1h, Supplementary figure 2e). CD4$^+$ and CD8$^+$ single positive (SP) T cells were present in normal ratios; however, both central and effector memory phenotypes were overrepresented while naïve T cells were still limited (Figure 1h, Supplementary figure 2f).

Thymus transplant recipient T cells have deficits in homeostatic expansion

T cell development in an MHC-mismatched thymus recipient is a complex scenario, where T cells restricted on donor MHCs must functionally recognise recipient MHC molecules in a way to both prevent autoimmunity and respond to foreign antigens. Previous studies using thymus xenografts suggested that positive selection occurs exclusively on MHCs expressed by the donor thymus and that the T cell receptor (TCR) repertoire of newly developed T cells must have sufficient cross-reactivity with host MHC molecules as they are released into circulation.[19] To test this in our system using donor thymus from the same species but different genetic backgrounds, we implanted cultured MHC-II deficient thymus from C57BL/6 mice to BALB/c *Foxn1*^{nu} mice to test whether recipient mice were able to reconstitute a CD4$^+$ SP T cell population, which requires binding with MHC-II for survival.[20] Recipient mice were able to produce CD4$^+$ SP T cells; however, the ratio of CD4$^+$ and CD8$^+$ SP T cells was substantially biased toward CD8$^+$ T cells (Figure 2a). It is possible that the fraction of CD4$^+$ T cells present maybe be selected on recipient MHC-II expressing populations that infiltrate into the MHC-II deficient thymus (which has also been suggested by the Davies lab[12]), as we detected infiltrating, host-derived (i.e. CD45.2$^+$), B cells, macrophages and dendritic cells (Figure 2b, Supplementary figure 3). These data suggest most thymic positive selection relies on MHC-II expressed by the implanted thymus. Once in the periphery, naïve T cells need continued TCR:MHC engagement for their survival over time and to expand under homeostatic proliferation in a lymphopenic environment.[21,22] To measure the

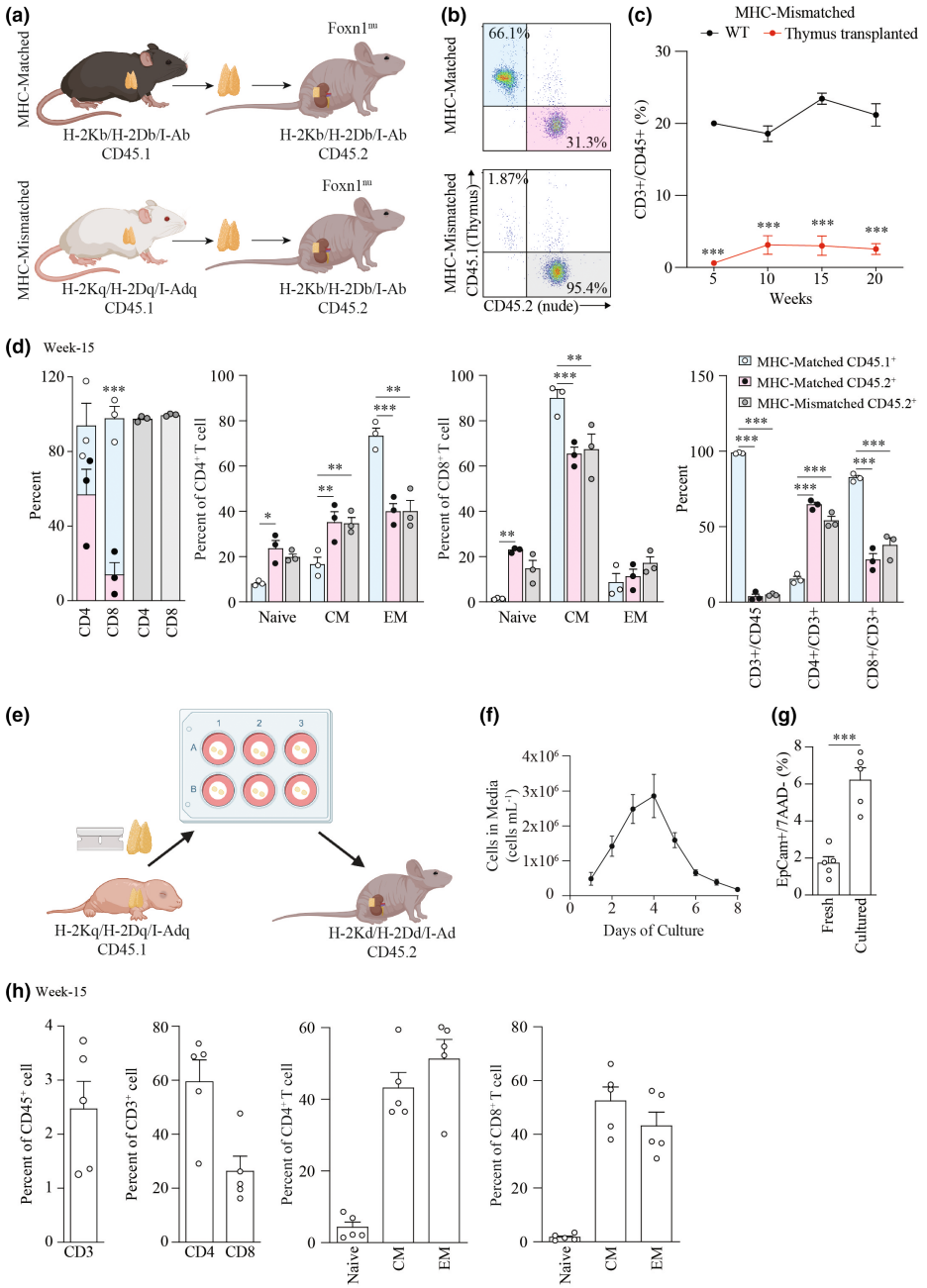

Figure 1. Thymus implants repopulate T cells in *Foxn1*^{nu} athymic recipient mice. **(a)** Experimental scheme for fresh thymus implants. **(b)** Representative scatter plots of peripheral blood collected from *Foxn1*^{nu} mice implanted with thymus and labelled with anti-CD3, CD45, CD4, CD8, CD44 and CD62L antibodies. Cells were analysed using FACS to compare the T cell populations derived from recipient mice or donor tissue (blue = T cells from donor thymus in MHC-Matched implant, pink = new T cells developed in the recipient after MHC-matched thymus implants, grey = new T cells developed in the recipient after MHC-Mismatched thymus implants). **(c)** Percentage of T cells/CD45+ cells in peripheral blood over time (weeks post post-implant) in MHC-mismatched thymus implantation compared to age-matched wild-type mice (N = 3 or 4 mice/group from 2 experiments; 2-way ANOVA $P < 0.001$; post-hoc ***$P < 0.001$). **(d)** FACS analysis of peripheral blood T cells, 15 weeks post thymus implant (CM: central memory T cells; EM: effector memory T cells; N = 3 or 4 mice/group from 3 experiments; one-way ANOVA; post-hoc *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). **(e)** Experimental scheme for cultured thymus implants using neonatal donor mice (aged 2–4 days). **(f)** Cellometer counts of cells in thymus culture media over time show peak at day 3 or 4 and decrease through day 8 (N = 9–16 cultures per day in 1 experiment). **(g)** TEC populations in acutely isolated and cultured thymus were analysed by FACS (N = 5 thymus per group from 1 experiment; $P < 0.001$, t-test). **(h)** Percentage of T cell subsets of total CD45^{+} cells in peripheral blood of *Foxn1*^{nu} athymic mice 15 weeks after cultured thymus implants (N = 5 mice per group from two experiments).

dynamics of T cells generated from a thymus implant and their ability to engage MHC molecules in the periphery, we set up competitive adoptive transfer assays between T cells generated from a thymus implant and T cells generated from typical thymopoiesis. We transferred equal numbers of thymus transplant-derived T cells (CD45.2; from the primary recipient 1°) and control T cells (CD45.1) into secondary *Foxn1*^{nu} mice recipients (2°) that either expressed MHC-II that matched the transplant itself (Figure 2c–e) or the primary transplant recipient (Figure 3a–e). Because we wanted to test the ability of each T cell compartment to expand, we transferred equal numbers of each compartment and not equal numbers of each isolated T cell subset (i.e. naïve, central memory and effector memory). For both experiments, the 1° recipients and the control mice were the same MHC haplotype (H-2Kb/H-2Db/I-Ab), which allowed us to determine whether the newly developed T cells in the 1° recipient would have a more robust expansion in a 2° recipient whose MHCs matched the genotype of the thymus transplant or the 1° recipient. When the MHC haplotype of the 2° recipient matched between 1° thymus recipient and control group, approximately 80% of both CD4^{+} and CD8^{+} SP T cells observed in the peripheral blood of the 2° *Foxn1*^{nu} recipients after adoptive transfer were CD45.1^{+} control-derived (Figure 2d). The remaining 20% or less of those T cells were CD45.2^{+}, originating from 1° thymus transplant recipient (Figure 2d). Additionally, the phenotype of control T cells was dynamic over the 12 weeks of homeostatic expansion, shifting from primarily naïve T cells to effector memory T cells (Figure 2e, Supplementary figure 4a). This transformation was not as apparent in CD45.2^{+} transplant-derived T cells where the dynamics remained stagnant (Figure 2e). When the MHC haplotype of the 2° recipient matched the thymus (Figure 3a), we detected an increase in the percentage of adoptively transferred cells from the 1° thymus recipient; however, at 15 weeks post transfer, they still were less than 40% compared to T cells transferred from control mice (Figure 3b). Again, T cells from the 1° recipient were less dynamic than control T cells (Figure 3c). Notably, in this scenario, both T cell compartments were enriched with central memory compared to the first scenario which was enriched with effector memory. This likely exemplifies the importance of other variables, such as the cytokine milieu, commensal antigens and dependence (or lack of) on TCR/MHC interactions for the homeostatic expansions of T cell subsets.[23] Overall, these data suggest that there is a role for peripheral MHC matching in the recipient and donor thymus regarding homeostatic expansion. However, even if matched, T cells developed from an implanted thymus fail to expand to normal levels. Expansion deficits may be because of either decreased T cell proliferation or increased cell apoptosis. To test this, T cells from control mice or from 1° implantation recipients were labelled using CFSE before adoptive transfer into 2° mice that MHC haplotype matched the thymus donor. We then assessed proliferation in peripheral blood samples with CFSE dilution as well as apoptosis and necrosis with 7AAD and Annexin V (Supplementary figure 3b). Surprisingly, on days 3 and 7 post-transfer, T cells from 1° thymus donors proliferated more robustly than control T cells but underwent increased apoptotic/necrotic cell death (Figure 3d and e, Supplementary figure 4b, c). These results suggested that T cells developing from an implanted thymus may have deficits in expansion because of rampant proliferation and cell death in the periphery.

IL-7-enhanced homeostatic proliferation of T cells in thymus-transplanted athymic mice

In the periphery, the T cell compartment is maintained by homeostatic mechanisms that rely in part on IL-7 and IL-15 signalling.[23,24] In a lymphopenic environment, whether it be during normal foetal development or adoptive T cell transfer/thymus implantation into a lymphopenic host, T cells rapidly expand and convert to a memory phenotype until T cell numbers are elevated and competition is established.[25,26] Expansion occurs under two mechanisms: a slower IL-7 dependent 'homeostatic proliferation' and a rapid IL-7 independent 'spontaneous proliferation' characterised by increased generation of memory/effector T cell.[27] Based on this, we hypothesized that limited naïve populations and total T cell numbers after thymus implantation are due to aberrant spontaneous proliferation and an inability to populate a proper T cell compartment. To test this, we co-implanted osmotic pumps containing 4 weeks of recombinant IL-7 (rIL-7; with a predicted release of 293.3 ng per day) alongside cultured thymus implants (Figure 4a). We further optimised our thymus implantation

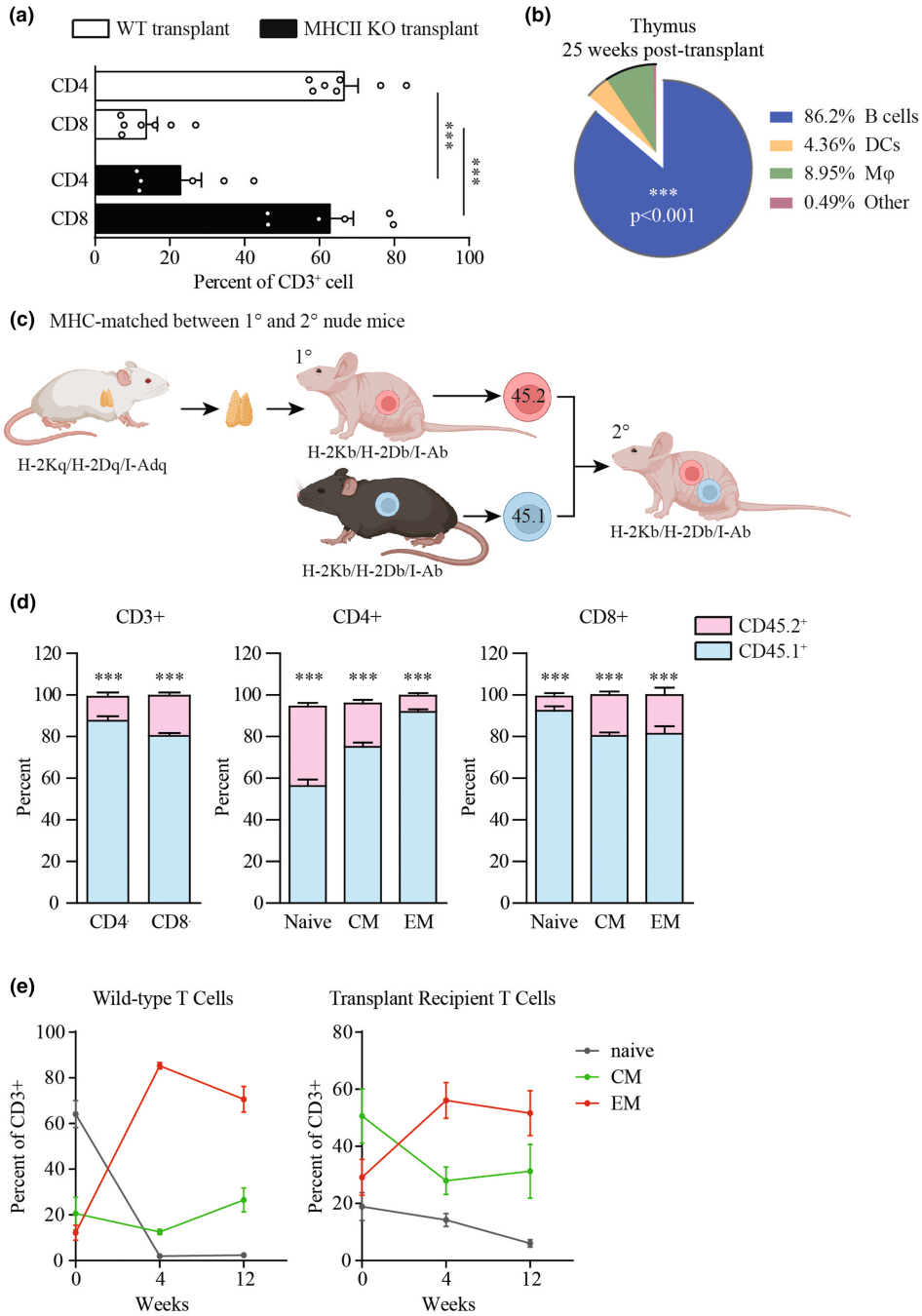

Figure 2. T cells educated by implanted thymus have expansion deficit in an MHC mis-matched periphery. **(a)** FACS analysis of peripheral blood T cells from *Foxn1*^{nu} athymic mice transplanted with wild-type (WT) or MHC-II knockout (KO) thymus ($N = 6$ or 7 mice per group from two experiments; two-way ANOVA for interaction, $P < 0.001$; post-hoc; ***$P < 0.001$ post-hoc). **(b)** FACS analysis of MHC-II+ recipient cells (gated on CD45.2) infiltrating recovered MHC-II KO thymus (CD45.1), 25 weeks post-transplant ($N = 6$ thymus from two experiments; ***$P < 0.001$, chi-squared). **(c)** Experimental scheme of competitive adoptive transfer of T cells. Cultured thymus was transplanted into *Foxn1*^{nu} athymic mice with mismatched MHC-haplotype. Newly generated T cells from primary transplants (1°) were combined 50:50 with T cells from control mice and transferred into 2° *Foxn1*^{nu} athymic mice which MHC haplotype matched the genetic background of the 1° recipients and control mice. **(d)** FACS analysis of peripheral blood taken from 2° mice 12 weeks after adoptive transfer. Data are represented as the percentage of T cells from 1° transplant recipients (CD45.2; pink) or control mice (CD45.1; blue; $N = 4$ mice/group from two experiments; two-way ANOVA; post-hoc ***$P < 0.001$). **(e)** The ratio of T cell subsets (Naïve T cells, CM = central memory T cells, EM = effector memory T cells) was analysed by FACS ($N = 4$ mice/group from two experiments; two-way repeated measures ANOVA; post-hoc ***$P < 0.001$).

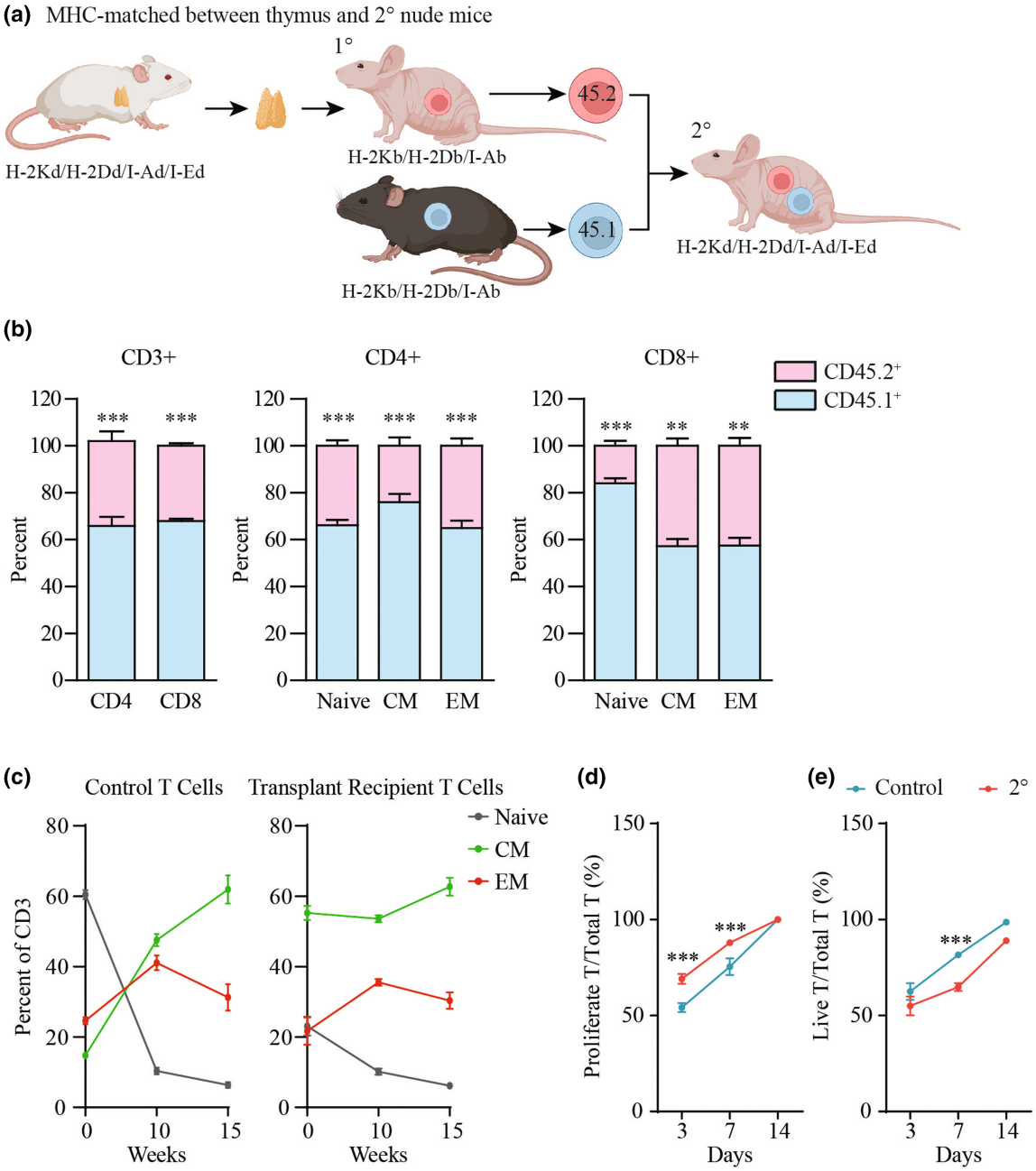

Figure 3. T cells educated by implanted thymus have expansion deficit in an MHC-matched periphery. **(a)** Experimental scheme of competitive adoptive transfer of T cells. Cultured thymus was transplanted to *Foxn1*^{nu} athymic mice with mismatched MHC-haplotype. Newly generated T cells from primary transplants (1°) were combined 50:50 with T cells from control mice and transferred to 2° *Foxn1*^{nu} athymic mice which MHC haplotype matched the donor thymus. **(b)** FACS analysis of peripheral blood taken from 2° mice 15 weeks after adoptive transfer. Data are represented as the percentage of T cells from 1° transplant recipients (CD45.2; pink) or control mice (CD45.1; blue; $N = 5$ mice/group from 1 experiment; two-way ANOVA; post-hoc $^{**}P < 0.01$, $^{***}P < 0.001$). **(c)** The ratio of T cell subsets (Naïve T cells, CM = central memory T cells, EM = effector memory T cells) was analysed by FACS ($N = 5$ mice/group from 1 experiment; two-way repeated measures ANOVA; post-hoc $^{***}P < 0.001$). **(d)** To compare T cell proliferation, T cells were stained with CFSE, and CFSE signal dilution on T cells was analysed using FACS ($N = 5$ mice/group from 1 experiment; two-way repeated measures ANOVA; post-hoc $^{***}P < 0.001$). **(e)** Peripheral blood leukocytes were stained using 7AAD and Annexin V and 7AAD^{-}AnnexinV^{-} CD3^{+} cells were defined as live T cells ($N = 5$ mice/group from 1 experiment; two-way ANOVA; post-hoc $^{***}P < 0.001$).

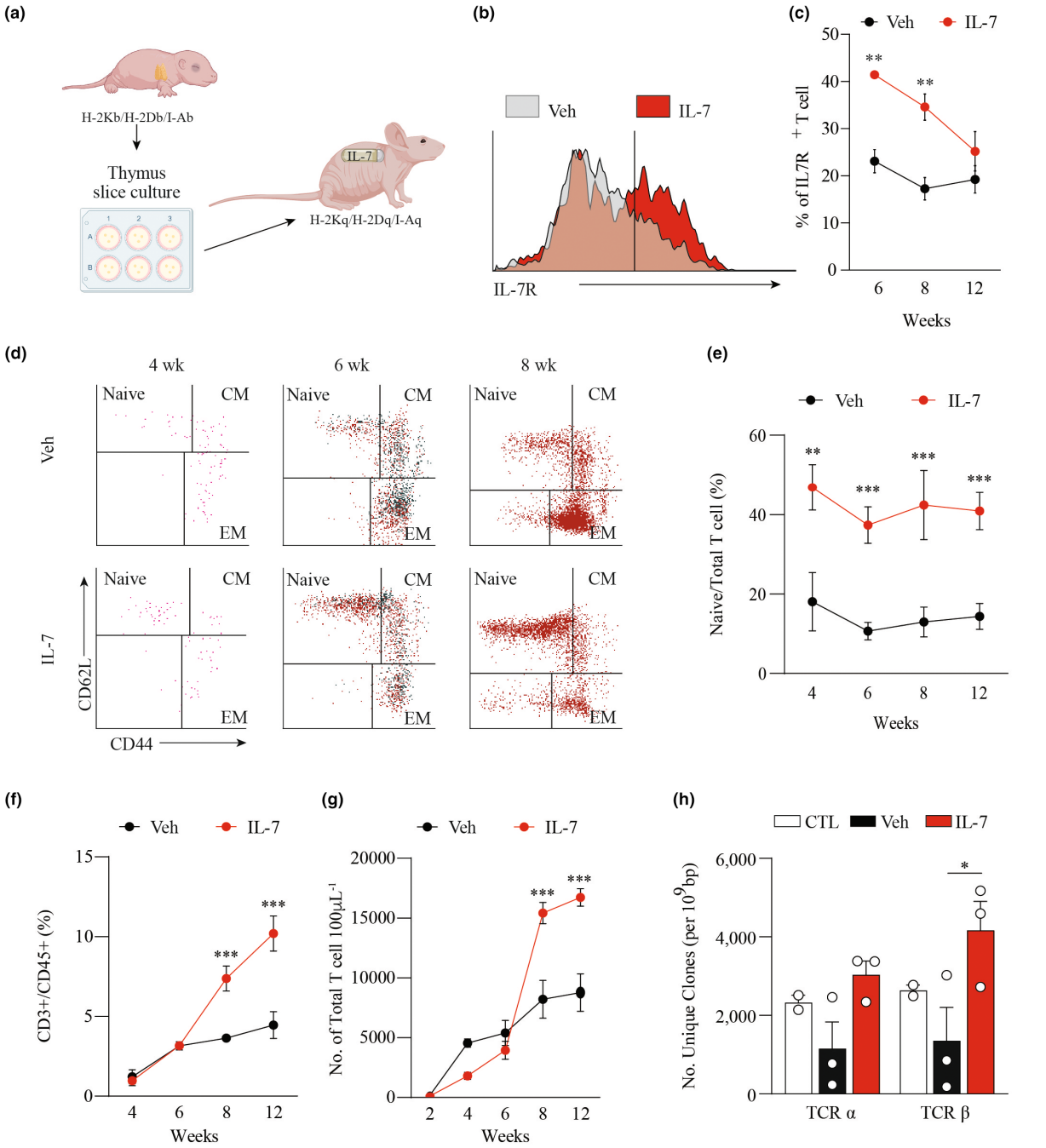

Figure 4. IL-7 enhanced T cell development in peripheral blood by increasing the population of naïve T cells. **(a)** Experimental scheme treating *Foxn1*^{nu} athymic mice with cultured thymus transplants and IL-7. *Foxn1*^{nu} athymic mice that received a cultured, MHC-mismatched thymus (intramuscularly) were implanted with a mini-osmotic pump containing PBS (Veh) or 5 μg of murine IL-7 in PBS. **(b, c)** To compare the homeostatic proliferated T cell population, peripheral leukocytes were stained using IL-7R antibody and the signal was analysed using FACS ($N = 4$ mice/group from 1 experiment; two-way repeated measures ANOVA; $^{**}P < 0.01$). **(d)** Peripheral blood collected from control or IL-7-treated mice transplanted with thymus was stained using anti-CD45.1, CD45.2, CD3, CD4, CD8 CD44 and CD62L antibodies and analysed by FACS. T cell subpopulations were analysed and compared between the control and IL-7-treated groups based on CD44 and CD62L expression. **(e–g)** Data are presented as CD44^{lo}CD62L^{hi} naïve populations per total cells (e), percentage of T cells per leukocytes **(f)** and absolute number of T cells in 100 μL of blood **(g)** on weeks 2, 4, 6, 8 and 12 after surgery ($N = 6$ mice/group from 2 experiments; two-way repeated measures ANOVA; $^{*}P < 0.05$, $^{**}P < 0.01$ post-hoc $^{***}P < 0.001$). **(h)** To compare TCR repertoire, the number of unique TCR clones was compared by RNA sequencing ($N = 3$ mice/group from 1 experiment; two-way repeated measures ANOVA; $^{*}P < 0.05$).

procedures to deliver cultured thymus intramuscularly. This more closely matched the clinical protocol, dramatically decreased the complexity of the surgery and yielded similar numbers to intrarenal implantation (Supplementary figure 5). In implanted mice treated with rIL-7, a higher percentage of newly developed T cells expressed the IL-7 receptor (IL-7R) rendering them more capable of homeostatic proliferation (Figure 4b and c, Supplementary figure 6a). Most importantly, implanted *Foxn1*^{nu} mice treated with rIL-7 developed typical percentages of naïve T cells in circulation (Figure 4c) and, unlike *Foxn1*^{nu} mice treated with vehicle, T cells in mice treated with rIL-7 continued to develop beyond 8 weeks post-transplant (Figure 4d and e). As expected, absolute T cell counts rapidly increased in vehicle-treated mice for the initial 6 weeks (likely because of rapid spontaneous proliferation), but T cell numbers continued to increase in rIL-7-treated mice and surpassed controls out to 12 weeks (Figure 4f and g). Importantly, rIL-7 increased the T cell repertoire by increasing the number of unique clones within the TCRβ gene (Figure 4h). Unlike in the blood, treating with systemic rIL-7 did not alter the number of developing T cells within the thymus transplant (Supplementary figure 6b, c). Overall, our data demonstrate that rIL-7 improves the development of naïve and total T cells in *Foxn1*^{nu} mice implanted with cultured thymus.

DISCUSSION

Thymus implantation is a recently approved therapy for patients born with congenital athymia. Recipients of MHC-unmatched donor thymus implants are able to generate a small T cell pool that is capable of recognising self-MHC as evidenced by the ability of implanted patients to clear infections post-transplant.[9] Although the benefits are clear, T cells developing after thymus implantation do not expand above 10% of a typical T cell compartment and patients often develop autoimmunity, identifying the need to improve thymus implantation procedures. In this study, we developed a model to study clinical thymus implantation using *Foxn1*^{nu} athymic mice. We first established that implanting an MHC-mismatched donor thymus led to the development of T cells that originated from the recipient. On the contrary, using T cells from an MHC-matched thymus for an adoptive transfer resulted in most T cells in the recipient being donor-derived. Using thymus implants from MHC-II deficient donors, we determined that the majority of T cell education after implantation occurs on MHC-II molecules expressed on the surface of cells within the donor thymus. It is likely that donor thymic epithelial cells (TECs) are the primary cells that facilitate positive selection of recipient T cells, as TECs are the highly specified cells responsible for normal thymic selection.[28,29] Alternatively, recipient APCs may migrate to the donor thymus in order to act as surrogate TECs and select recipient T cells with proper self-MHC restricted TCRs. However, the decreased number of CD4^{+} T cells in MHC-II KO transplants would suggest that APCs are not the primary cell responsible for T cell selection after thymus implantation. In MHC-II deficient implants, we detected small populations of recipient-derived cells expressing MHC-II, but their role in positive or negative selection has yet to be tested. Although genetics are easy to control in the experimental murine setting, MHC genetics is more complicated in humans and thymus matching is not considered under clinical implantation protocols.[7] To prevent donor-derived GVHD, thymus is cultured to remove donor T cells. With this is mind, we developed and optimised a protocol to isolate thymus from neonatal mice and culture for 8 days. This system would permit additional manipulation of the thymus for further optimization or to target other indications (e.g. to promote tolerance in autoimmune diseases like diabetes and multiple sclerosis[30,31]). We further optimised the implantation procedure from inserting thymus under the kidney capsule to implanting into the muscle, which aligns with the clinical protocol, is less invasive, and dramatically reduces surgical times and potential failures.

Our model demonstrated that, like that observed after clinical thymus implantation, recipients developed a small T cell compartment enriched with memory T cells. T cell development after implantation plateaus in both humans (~12 months post-implantation) and mice (~8 weeks post-implantation), thus never reaching a complete T cell compartment.[5] Why T cell development and/or expansion halts is unknown, although there are a few possible explanations. For one, TECs in the thymus graft may die or be actively rejected, similar to what is proposed after allogeneic bone marrow transplantation.[32] Another possibility could be a dysregulated peripheral environment. For example, *Foxn1*^{nu} athymic mice and individuals with 22q11.2

deletion syndrome have dysregulated cytokine production.[33,34] This could impact the expansion of recent thymic emigrants in the periphery, although in our adoptive transfer competition assays, the control T cell compartment outperformed the T cell compartment educated on a thymus transplant. An additional possibility could be an aberrant interaction between this peripheral environment and T cells educated on a thymus transplant.

T cells undergo expansion to fill an empty niche. Experimentally, this is apparent after an adoptive transfer of T cells in a lymphopenic host; however, similar steps have been suggested to occur after natural T cell development before the T cell compartment reaches completeness.[35,36] Naïve T cells emigrating from the thymus rapidly expand and lose their naïve phenotype until competition is established in the T cell compartment. It is possible that the output of naïve T cells educated by an implanted thymus is so limited that competition for resources in the peripheral T cell compartment is never established. For naïve T cells, the compartment is maintained through self-antigens and IL-7,[17,37] and both are limited resources. When we delivered rIL-7 with implanted thymus, this promoted the establishment of naïve T cell compartments and boosted the total number of T cells in circulation. IL-7 plays an important role in the development and function of numerous lymphoid and myeloid populations.[38] Further studies are needed to conclude whether rIL-7 is acting directly on naïve T cells to promote expansion or whether rIL-7 is also indirectly promoting the expansion of naïve T cells through an intermediate cell type.

rIL-7 has been used with success in clinical trials to elevate adaptive immunity for indications ranging from cancers to viral infections.[39] In two Phase I dose escalation studies, patients with refractory cancers treated with recombinant human IL-7 (rhIL-7) increased $CD4^+$ and $CD8^+$ T cell counts with increased total numbers of recent thymic emigrants and increased TCR diversity.[40,41] Similarly, increases in T cell counts were observed in patients with metastatic breast cancer treated with rhIL-7.[42] Patients with metastatic castration-resistant prostate cancer tolerated rhIL-7 well and, although not powered to detect clinical efficacy, patients treated with rhIL-7 had elevated antigen-specific humoral and T cell responses.[43] In a placebo-controlled, double-blind study, rhIL-7 elevated circulating T cell counts 3–4 fold in lymphopenic patients suffering from sepsis.[44] Likewise, rhIL-7 has shown success in boosting adaptive immunity against other infections including HIV, JC virus and more recently, SARS-CoV2.[45–49] Although rhIL-7 has been clinically well tolerated, several studies have suggested uncontrolled IL-7 can lead to hyper-proliferation of T cells which can have implications for chronic inflammatory disease or lymphocytic leukaemia.[50–52] This suggests that further studies are needed to fine-tune the delivery of IL-7 to limit the target tissue, dosage and duration. Notably, our experiments used an acute, 4-week treatment dose to establish the naïve T cell compartment and promote peripheral T cell cellularity and completion.

In this study, we developed a model of thymus implantation that closely recapitulates results in clinical implantation for infants born with congenital athymia. We provide a tool that can be manipulated to further investigate how implanted thymus educates recipient T cells. We determined that rIL-7 promoted T cell development and established a greater naïve T cell compartment with an increased TCR repertoire. Further work is needed to test the function and diversity of the T cell compartment; however, our data suggest that exogenous delivery of IL-7 could enhance T cell recovery after thymus implantation in patients with congenital athymia.

METHODS

Mice

Male C57BL/6 (B6) CD45.1, B6 *Foxn1*^{nu} ($CD45.2^+$), FVB, BALB/c and B6 MHC-II knockout were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Male CAnN.Cg-*Foxn1*^{nu}, (BALB/c *Foxn1*^{nu}) were obtained from Charles River (Wilmington, MA, USA). All mice were housed under normal conditions. All animal experiments were conducted with approval from the Duke Institutional Animal Care and Use Committee and were following the US National Research Council's Guide for the Care and Use of Laboratory Animals, the US Public Health Service's Policy on Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals.

Thymus culture and implantation

Donor thymus was harvested under aseptic conditions from p2 to p5 donor mice after euthanasia (B6 CD45.1, FVB, MHC-II KO) and transferred back to the lab in thymus organ media on ice. Thymus organoid media contains Hams F-12 (Gibco, Gaithersburg, MD, USA), 2.5% HEPES (Gibco, 1 M), 10% heat-inactivated FBS (Gibco), 1% L-glutamine

(Gibco 200 mM) and 1% antibiotic-antimycotic (100X). Thymuses were sliced using a razor blade to separate the two lobes and each lobe was sliced into at least two pieces. All slices from one thymus were placed in one 6-well plate containing a piece of filter paper (Millipore Sigma, Bedford, MA, USA) lying on a piece of Surgifoam absorbable gelatin sponge (Ethicon, Duke Pharmacy, Durham, NC, USA) for 7–15 days in media mimicking the culturing of human thymus for implantation as previously described.[53] The media was changed daily, and number of cells in media and their viability were assessed on a Cellometer Auto 2000 (Nexcelom, Lawrence, MA, USA) after washing with MACs buffer and centrifuging at ~1300 rpm. After culturing, slices of thymus were transferred in sterile conditions to the vivarium and transplanted intrarenally, under the kidney capsule, into 4-week-old male *Foxn1*^{nu} mice. For intramuscular thymus implantation, incision was made on supraspinatus muscle and incision was opened up to scapula bone to make muscle pocket. Thymus slices were pushed into pocket space using forceps incision was closed by suture.

Competitive adoptive transfer of T cells

Peripheral blood mononuclear cells (PBMCs) were pooled from spleens, lymph nodes and blood from either a fresh thymus implant recipient 25 weeks post-implantation or B6 CD45.1 mouse. T cells were isolated using a MAC pan-T-cell isolation kit II (Miltenyi Biotec, Auburn, CA, USA). T cells were washed with MACs buffer, counted on the Cellometer (Nexcelom Bioscience LLC, Lawrence, MA, USA), and combined at equal numbers in sterile PBS: 1.9×10^6 per mouse for 3.8×10^6 total T cells in 200 μL per injection. T cell injections were administered *via* the tail vein to B6 *Foxn1*^{nu} mice. Peripheral blood was analysed by flow cytometry at 4 and 12 weeks after adoptive transfer.

IL-7 treatment using an osmotic pump

Before implant, osmotic pumps (DURECT Corporation, Cupertino, CA, USA) were primed in 0.9% NaCl saline at 37°C for 2 days. On the day of thymus implantation, the pump was filled with 10 μg of IL-7 in 180 μL of PBS using a syringe and implanted in mouse subcutaneously after thymus implantation. Peripheral blood samples were collected and further processed for flow cytometry.

Peripheral blood T cell analysis by flow cytometry

Murine blood was collected *via* the submandibular vein into sodium heparin-coated tubes (Bectin, Dickinson and Company, San Jose, CA, USA). Red blood cells were lysed using ACK lysis buffer (VWR, Radnor, PA, USA). The remaining cells were washed with PBS, resuspended in FACS buffer (PBS + 2% FBS) and stained with antibodies for 30 min at 4°C. Antibodies for the T cell immunophenotyping panel were purchased from BD Biosciences: PerCP-Cy5.5 CD45.1 (Clone: A20), PE-Cy7 CD44 (Clone: IM7), APC CD3e (Clone: 145-2C11), APC-H7 CD4 (Clone: GK1.5), V450 CD45.2 (Clone: 104), V500 CD8 (Clone: 53–6.7) and BV605 CD62L (Clone: MEL-14), AF488 CD45R/B220 (Clone: RA3-6B2), PE CD45b (Clone: DX5), Alexa Fluor-700 CD11c (Clone: N418), APC-Cy7 CD11b (Clone: M1/70) and IL-7R (Clone: SB/199). Samples were acquired on a FACsLyric™ cytometer with FACSuite™ software (BD Biosciences); results were analysed using FACSuite™ or FlowJo™ v10 software (BD, Ashland, OR, USA).

TCR sequencing

Twelve weeks post-thymic implantation, cells were collected and pooled from spleens, lymph nodes and blood. After RBC lysis using ACK lysis buffer, T cells were isolated using a MojoSort™ Mouse T Cell Isolation Kit (BioLegend, San Deigo, CA, USA). RNA samples were collected from T cells using NucleoSpin® RNA (TAKARA BIO INC., Kusatsu, Shiga, Japan). TCR cDNA libraries were generated using the SMARTer Mouse TCR a/b Profiling Kit (TAKARA BIO INC., Kusatsu, Shiga, Japan) according to the manufacturer's protocols. Libraries were sequenced using the MiSeq v3 platform (Illumina, San Deigo, CA, USA) to produce 301-bp paired-end reads. The yield for each sample was $1.07 \times 10^9 \pm 0.12 \times 10^9$ bp. Sequencing data were processed using MiXCR (v3.0.13) and TCR clones with at least 10 reads for each sample were quantified. Sequencing data generated and presented in Figure 4 are deposited in the Gene Expression Omnibus (GEO) GSE246565.

Thymus tissue analysis by flow cytometry

Thymus was dissected from the kidney capsule or muscle pocket under a dissecting scope and digested in HBSS (Thermo Fisher Science, Waltham, MA, USA) with 0.6 mg mL^{-1} collagenase D (#11088882001 MilliporeSigma, Burlington, MA, USA) and 50 U mL^{-1} DNase I (#11284932001 MilliporeSigma, Burlington, MA, USA) for 30 min at 37°C with shaking. After digestion, tissue was passed through a 70-μm cell strainer and washed with FACS buffer. Cells were stained using following antibodies from BD Biosciences: AF488 CD45R/B220 (Clone: RA3-6B2), V450 CD45.2 (Clone 104), V500 CD3 (Clone: 500A2), BV605 CD19 (Clone: 1D3), PE CD49b (#Clone: DX5), PE-Cy7 I-A/I-E (Clone: M5/114.15.2), APC F4/80 (Clone: BM8), Alexa Fluor-700 CD11c (Clone: N418) and APC-Cy7 CD11b (Clone: M1/70).

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AUTHOR CONTRIBUTIONS

Hyunjung Min: Conceptualization; formal analysis; investigation; methodology; writing – original draft; writing – review and

editing. **Laura A Valente:** Conceptualization; formal analysis; investigation; methodology; writing – original draft; writing – review and editing. **Li Xu:** Investigation; methodology; writing – review and editing. **Shane M O'Neil:** Formal analysis; writing – review and editing. **Lauren R Begg:** Formal analysis; investigation; methodology; writing – review and editing. **Joanne Kurtzberg:** Conceptualization; funding acquisition; writing – review and editing. **Anthony J Filiano:** Conceptualization; formal analysis; funding acquisition; investigation; methodology; project administration; supervision; writing – original draft; writing – review and editing.

CONFLICT OF INTEREST

Joanne Kurtzberg and Anthony Filiano have intellectual property licensed to Cryocell. Joanne Kurtzberg receives salary support from Enzyvant for clinical manufacturing of thymus for implantation into patients with congenital athymia.

DATA AVAILABILITY STATEMENT

TCR sequencing data used in this study are openly available under GEO GSE246565. All additional raw data that support the findings of this study are available from the corresponding author upon reasonable request.

REFERENCES

1. Davies E. Immunodeficiency in DiGeorge syndrome and options for treating cases with complete athymia. *Front Immunol* 2013; **4**: 322.
2. Markert ML, Hummell DS, Rosenblatt HM *et al*. Complete DiGeorge syndrome: Persistence of profound immunodeficiency. *J Pediatr* 1998; **132**: 15–21.
3. Rice HE, Skinner MA, Mahaffey SM *et al*. Thymic transplantation for complete DiGeorge syndrome: Medical and surgical considerations. *J Pediatr Surg* 2004; **39**: 1607–1615.
4. Howley E, Davies EG, Kreins AY. Congenital athymia: Unmet needs and practical guidance. *Ther Clin Risk Manag* 2023; **19**: 239–254.
5. Davies EG, Cheung M, Gilmour K *et al*. Thymus transplantation for complete DiGeorge syndrome: European experience. *J Allergy Clin Immunol* 2017; **140**: 1660–1670.e1616.
6. Lee JH, Markert ML, Hornik CP *et al*. Clinical course and outcome predictors of critically ill infants with complete DiGeorge anomaly following thymus transplantation. *Pediatr Crit Care Med* 2014; **15**: e321–e326.
7. Markert ML, Devlin BH, Chinn IK, McCarthy EA, Li YJ. Factors affecting success of thymus transplantation for complete DiGeorge anomaly. *Am J Transplant* 2008; **8**: 1729–1736.
8. Markert ML, Gupton SE, McCarthy EA. Experience with cultured thymus tissue in 105 children. *J Allergy Clin Immunol* 2022; **149**: 747–757.
9. Markert ML, Devlin BH, McCarthy EA. Thymus transplantation. *Clin Immunol* 2010; **135**: 236–246.
10. Markert ML, Devlin BH, Chinn IK, McCarthy EA. Thymus transplantation in complete DiGeorge anomaly. *Immunol Res* 2009; **44**: 61–70.
11. Markert ML, Devlin BH, Alexieff MJ *et al*. Review of 54 patients with complete DiGeorge anomaly enrolled in protocols for thymus transplantation: Outcome of 44 consecutive transplants. *Blood* 2007; **109**: 4539–4547.
12. Kreins AY, Bonfanti P, Davies EG. Current and future therapeutic approaches for thymic stromal cell defects. *Front Immunol* 2021; **12**: 655354.
13. MacNabb BW, Rothenberg EV. Speed and navigation control of thymocyte development by the fetal T-cell gene regulatory network. *Immunol Rev* 2023; **315**: 171–196.
14. Benoist C, Mathis D. Selection for survival? *Science* 1997; **276**: 2000–2001.
15. Marrack P, Mitchell T, Bender J *et al*. T-cell survival. *Immunol Rev* 1998; **165**: 279–285.
16. Min B, Yamane H, Hu-Li J, Paul WE. Spontaneous and homeostatic proliferation of CD4 T cells are regulated by different mechanisms. *J Immunol* 2005; **174**: 6039–6044.
17. Tan JT, Dudl E, LeRoy E *et al*. IL-7 is critical for homeostatic proliferation and survival of naive T cells. *Proc Natl Acad Sci USA* 2001; **98**: 8732–8737.
18. Nehls M, Pfeifer D, Schorpp M, Hedrich H, Boehm T. New member of the winged-helix protein family disrupted in mouse and rat nude mutations. *Nature* 1994; **372**: 103–107.
19. Nauman G, Borsotti C, Danzl N *et al*. Reduced positive selection of a human TCR in a swine thymus using a humanized mouse model for xenotolerance induction. *Xenotransplantation* 2020; **27**: e12558.
20. Walsh JT, Hendrix S, Boato F *et al*. MHCII-independent $CD4^+$ T cells protect injured CNS neurons via IL-4. *J Clin Invest* 2015; **125**: 699–714.
21. Ernst B, Lee DS, Chang JM, Sprent J, Surh CD. The peptide ligands mediating positive selection in the thymus control T cell survival and homeostatic proliferation in the periphery. *Immunity* 1999; **11**: 173–181.
22. Labrecque N, Whitfield LS, Obst R, Waltzinger C, Benoist C, Mathis D. How much TCR does a T cell need? *Immunity* 2001; **15**: 71–82.
23. Surh CD, Sprent J. Homeostasis of naive and memory T cells. *Immunity* 2008; **29**: 848–862.
24. Ellestad KK, Lin J, Boon L, Anderson CC. PD-1 controls tonic signaling and lymphopenia-induced proliferation of T lymphocytes. *Front Immunol* 2017; **8**: e1289.
25. Stockinger B, Barthlott T, Kassiotis G. The concept of space and competition in immune regulation. *Immunology* 2004; **111**: 241–247.
26. Dummer W, Ernst B, LeRoy E, Lee D, Surh C. Autologous regulation of naive T cell homeostasis within the T cell compartment. *J Immunol* 2001; **166**: 2460–2468.
27. Min B, Foucras G, Meier-Schellersheim M, Paul WE. Spontaneous proliferation, a response of naive CD4 T cells determined by the diversity of the memory cell repertoire. *Proc Natl Acad Sci USA* 2004; **101**: 3874–3879.

28. Anderson G, Takahama Y. Thymic epithelial cells: Working class heroes for T cell development and repertoire selection. *Trends Immunol* 2012; **33**: 256–263.
29. Klein L, Kyewski B, Allen PM, Hogquist KA. Positive and negative selection of the T cell repertoire: What thymocytes see (and don't see). *Nat Rev Immunol* 2014; **14**: 377–391.
30. Wu L, Li N, Zhang M *et al*. MHC-mismatched mixed chimerism augments thymic regulatory T-cell production and prevents relapse of EAE in mice. *Proc Natl Acad Sci USA* 2015; **112**: 15994–15999.
31. Zhang M, Racine JJ, Lin Q *et al*. MHC-mismatched mixed chimerism restores peripheral tolerance of noncross-reactive autoreactive T cells in NOD mice. *Proc Natl Acad Sci USA* 2018; **115**: E2329–E2337.
32. Muller-Hermelink HK, Sale GE, Borisch B, Storb R. Pathology of the thymus after allogeneic bone marrow transplantation in man. A histologic immunohistochemical study of 36 patients. *Am J Pathol* 1987; **129**: 242–256.
33. Aresvik DM, Lima K, Overland T, Mollnes TE, Abrahamsen TG. Increased levels of interferon-inducible protein 10 (IP-10) in 22q11.2 deletion syndrome. *Scand J Immunol* 2016; **83**: 188–194.
34. Kur-Piotrowska A, Kopcewicz M, Kozak LP, Sachadyn P, Grabowska A, Gawronska-Kozak B. Neotenic phenomenon in gene expression in the skin of Foxn1-deficient (nude) mice – a projection for regenerative skin wound healing. *BMC Genomics* 2017; **18**: 56.
35. Le Campion A, Bourgeois C, Lambolez F *et al*. Naive T cells proliferate strongly in neonatal mice in response to self-peptide/self-MHC complexes. *Proc Natl Acad Sci USA* 2002; **99**: 4538–4543.
36. Min B, McHugh R, Sempowski GD, Mackall C, Foucras G, Paul WE. Neonates support lymphopenia-induced proliferation. *Immunity* 2003; **18**: 131–140.
37. Schluns KS, Kieper WC, Jameson SC, Lefrancois L. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. *Nat Immunol* 2000; **1**: 426–432.
38. Chen D, Tang TX, Deng H, Yang XP, Tang ZH. Interleukin-7 biology and its effects on immune cells: Mediator of generation, differentiation, survival, and homeostasis. *Front Immunol* 2021; **12**: 747324.
39. Mackall CL, Fry TJ, Gress RE. Harnessing the biology of IL-7 for therapeutic application. *Nat Rev Immunol* 2011; **11**: 330–342.
40. Rosenberg SA, Sportes C, Ahmadzadeh M *et al*. IL-7 administration to humans leads to expansion of $CD8^+$ and $CD4^+$ cells but a relative decrease of $CD4^+$ T-regulatory cells. *J Immunother* 2006; **29**: 313–319.
41. Sportes C, Hakim FT, Memon SA *et al*. Administration of rhIL-7 in humans increases in vivo TCR repertoire diversity by preferential expansion of naive T cell subsets. *J Exp Med* 2008; **205**: 1701–1714.
42. Tredan O, Menetrier-Caux C, Ray-Coquard I *et al*. ELYPSE-7: A randomized placebo-controlled phase IIa trial with CYT107 exploring the restoration of $CD4^+$ lymphocyte count in lymphopenic metastatic breast cancer patients. *Ann Oncol* 2015; **26**: 1353–1362.
43. Pachynski RK, Morishima C, Szmulewitz R *et al*. IL-7 expands lymphocyte populations and enhances immune responses to sipuleucel-T in patients with metastatic castration-resistant prostate cancer (mCRPC). *J Immunother Cancer* 2021; **9**: e002903.
44. Francois B, Jeannet R, Daix T *et al*. Interleukin-7 restores lymphocytes in septic shock: The IRIS-7 randomized clinical trial. *JCI Insight* 2018; **3**: e98960.
45. Laterre PF, Francois B, Collienne C *et al*. Association of Interleukin 7 immunotherapy with lymphocyte counts among patients with severe coronavirus disease 2019 (COVID-19). *JAMA Netw Open* 2020; **3**: e2016485.
46. Levy Y, Lacabaratz C, Weiss L *et al*. Enhanced T cell recovery in HIV-1-infected adults through IL-7 treatment. *J Clin Invest* 2009; **119**: 997–1007.
47. Patel A, Patel J, Ikwuagwu J. A case of progressive multifocal leukoencephalopathy and idiopathic $CD4^+$ lymphocytopenia. *J Antimicrob Chemother* 2010; **65**: 2697–2698.
48. Sereti I, Dunham RM, Spritzler J *et al*. IL-7 administration drives T cell-cycle entry and expansion in HIV-1 infection. *Blood* 2009; **113**: 6304–6314.
49. Sereti I, Estes JD, Thompson WL *et al*. Decreases in colonic and systemic inflammation in chronic HIV infection after IL-7 administration. *PLoS Pathog* 2014; **10**: e1003890.
50. Nemoto Y, Kanai T, Takahara M *et al*. Bone marrow-mesenchymal stem cells are a major source of interleukin-7 and sustain colitis by forming the niche for colitogenic CD4 memory T cells. *Gut* 2013; **62**: 1142–1152.
51. Churchman SM, Ponchel F. Interleukin-7 in rheumatoid arthritis. *Rheumatology (Oxford)* 2008; **47**: 753–759.
52. Silva A, Laranjeira AB, Martins LR *et al*. IL-7 contributes to the progression of human T-cell acute lymphoblastic leukemias. *Cancer Res* 2011; **71**: 4780–4789.
53. Markert M, McCarthy E, Chinn I, Hale L. Thymus gland pathology: Clinical, diagnostic, and therapeutic features. 2008.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.