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Perspective

Rebuilding and rebooting immunity with stem cells

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SUMMARY

Advances in modern medicine have enabled a rapid increase in lifespan and, consequently, have highlighted the immune system as a key driver of age-related disease. Immune regeneration therapies present exciting strategies to address age-related diseases by rebooting the host's primary lymphoid tissues or rebuilding the immune system directly via biomaterials or artificial tissue. Here, we identify important, unanswered questions regarding the safety and feasibility of these therapies. Further, we identify key design parameters that should be primary considerations guiding technology design, including timing of application, interaction with the host immune system, and functional characterization of the target patient population.

MOTIVATIONS FOR REBUILDING AND REBOOTING IMMUNITY IN AGING

Over the last century, advances in modern medicine have enabled a rapid increase in lifespan, pushing our bodies far past the boundaries of natural selection pressures. With this demographic shift, it has become apparent that our immune system, which circulates between tissues and integrates body systems, is a weak link in the aging process.[1–3] The immune system is increasingly characterized as the driver of age-related diseases, including autoimmune disease, cardiovascular disease, neurodegenerative disease, increased risk of infection, and cancer.[4] This phenomenon, known as immunosenescence, was first defined by Roy Walford in 1964 and describes the decline, remodeling, and dysfunction of innate and adaptive immunity with aging.[5,6] In an aging society where increased immune function in the elderly would improve health span and greatly reduce the rising healthcare burden, stem cell-based cell and tissue therapeutic approaches to counteract immunosenescence and age-related immune impairment have gained traction.[2]

At present, there exist two schools of thought: those that focus on rejuvenating primary lymph tissue, namely the bone marrow and thymus, and those that focus on rebuilding the immune compartment directly by providing artificial niches for T cell production or injecting *in vitro*-derived immune cells.[7] Tissue-based thymus regeneration technologies include stimulating expansion of host thymic tissue, injection of hydrogel-based scaffolds to support T cell generation, transplantation of blood stem cells or progenitor T cells, and transplantation of pluripotent stem cell (PSC)-derived thymic tissue. Alternatively, direct *in vitro* generation of immune cells uses emerging engineered thymic niche technologies to produce mature T cells for direct supplementation of the peripheral immune compartment. Across these approaches, the theme is clear: our knowledge of stem and progenitor cells and their potential use in counteracting the effects of age-related immune impairment is the key to challenging our current understanding of healthspan. However, there remain important, unanswered questions regarding the various risks associated with modulating the natural evolution of immunity through aging.

In this perspective, we highlight the biological models of immune system aging and regeneration, which have laid the foundational knowledge of immune system manipulation. Furthermore, we will discuss the strengths, limitations, and clinical relevance of emerging technologies that aim to regenerate the aging immune system. Finally, we will suggest strategies to expand technologies to address key questions in the application of immune regeneration therapies in the context of aging and disease.

Immune system aging is a dynamic, progressive phenomenon

It remains unclear what specific molecular or cellular events initiate immune system deterioration and trigger the cascade of organismal aging. Age-related decline in immunity is in part catalyzed by a systemic increase in proinflammatory markers, termed "inflammaging," which is subsequently mirrored by an increase in immunosuppressive activity via generation of T regulatory cells (T_{regs}) and anergic T cells.[1,8,9] Age-related immunosuppressive activity leads to age-related diseases, including poor vaccination outcomes, increased risk of infection, and malignancies.[10–13] Similarly, the deleterious impact of decreased immunity has been well studied in patients who receive lifelong immunosuppression post-organ transplantation. Immunosuppression patients often present with severe complications later in life due to the side effects of suppressed immune responses,[14] including an increased risk of cancer,[15] infections,[16] autoimmune disease,[17] hypertension,[18] neurotoxicity,[19] and metabolic disorders,[20,21] among others. These patients represent a defined cohort with accelerated loss of immune function compared with individuals exhibiting age-related decreases in immunity.[22]

Age-related defects in adaptive immunity also play a major role in immune impairment-driven disease. Here, we focus on age-related defects in T cell immunity as an early driver of immune impairment. For further information on the age-related

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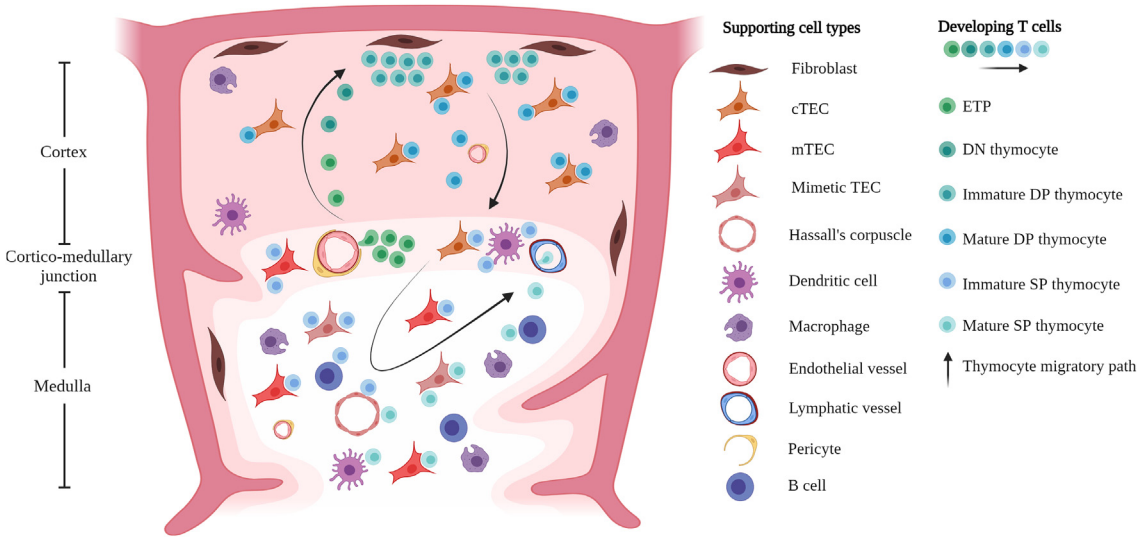

Figure 1. T cell development and selection are guided within the thymus microenvironment by spatiotemporal signals and interactions with supporting stromal, epithelial, and hematopoietic cells

Hematopoietic stem cells home from the bone marrow to the thymus and enter at the cortico-medullary junction as early thymic progenitors (ETP). These cells migrate toward the outer edge of the capsule, where they receive Notch signals, among other signals, from cortical thymic epithelial cells (cTEC) and fibroblasts to initiate the T cell developmental transcription factor program and become double negative (DN) thymocytes. At the outer edge of the cortex, thymocytes rearrange their T cell receptor (TCR) β-chain, upregulate CD4 and CD8 surface receptors to become double-positive (DP) thymocytes, and undergo rapid proliferation. Immature DP thymocytes with successfully rearranged β chains then rearrange their TCR α chain and begin migrating back toward the medulla as mature DP thymocytes, where they receive positive selection signals from cTECs to test their TCR. As DP thymocytes reach the medulla, they downregulate either CD4 or CD8 to become immature single-positive (SP) CD4 or CD8 thymocytes, where they then receive negative selection signals from medullary thymic epithelial cells (mTEC), mimetic thymic epithelial cells (TEC), dendritic cells, fibroblasts, and B cells. SP thymocytes that pass negative selection upregulate mature naive T cell markers and egress to the periphery as mature SP T cells.

impact of B cells on immune impairment, we refer readers to the following reviews.[23–26] The progressive decline of adaptive immune function is initiated by thymic involution, an age-related process by which thymus tissue progressively turns to adipose tissue. In healthy thymus, spatiotemporal developmental signals guide development of hematopoietic stem cells (HSCs) homing from the bone marrow to mature T cells that have been carefully selected to remove autoreactive cells (Figure 1). Thymic involution catalyzes major changes in the peripheral T cell compartment, resulting in a marked decrease in naive T cell generation and altered ratios of naive T cell subsets released into the periphery[27,28] (Figures 2A and 2B). This phenomenon has been shown to initiate surprisingly early in development, with declines in thymic cellularity and size, as well as adipogenic activity of mesenchymal cells, seen in infants under the age of one.[29,30] After one year of age, thymus activity, defined by volume of active, non-adipogenic thymus tissue, declines at a rate of 2%–3% per year until middle age when thymic involution slows to a rate of 1% per year, leading to significantly reduced activity in individuals 50–65 years of age.[31–33] However, even with significantly reduced T cell output, the aged thymus continues to contribute to immune function, as demonstrated by a recent retrospective study on aged thymectomy patients discussed below. Thymic involution is also accelerated during periods of stress, such as during puberty, pregnancy, or infection,[34–37] which has been shown in clinical studies to be linked to increased biological age.[38] In sum, thymic involution significantly pre-dates metabolic and inflammatory age-related changes arising in middle-aged individuals and acts as an early driver of age-related immune impairment (Figure 2C).

The decline in recent thymic emigrants throughout life coupled with recurrent exposure to pathogens significantly remodels T cell receptor (TCR) diversity in the peripheral T cell compartment (Figure 2C). During early fetal and postnatal development, the thymus is highly active, generating approximately 4×10^{11} T cells with an average TCR diversity of 10^{10} TCRs within the first year of life.[39,40] Exposure to antigens remodels an individual's TCR profile to reflect their environmental pathogens and confer adaptive immunity to previously encountered pathogens.[41] These changes allow an organism to rapidly mount an immune response against previously encountered pathogens to clear infections. However, with age, the thymus gradually undergoes involution, significantly reducing the output of naive T cells. The remaining T cells become increasingly reactive toward routinely encountered pathogens. This is best exemplified by exposure to latent viruses, such as Epstein-Barr virus (EBV), which can periodically leave dormancy and repeatedly activate T cells with TCR specificity toward a specific antigen.[42–45] This results in decreased TCR diversity and a lack of naive T cells available to respond to new exposures, as well as an exhausted T cell compartment against the latent virus. The dramatic decrease in T cell diversity with age is captured in recent single-cell TCR sequencing studies on elderly individuals, which demonstrate dramatic clonal expansion and decreased TCR diversity with age.[39,41,45] Ultimately, decreased naive T cell output, along with age-related chronic inflammation and metabolic changes, result in alterations in the peripheral T cell compartment, which may limit immune responses and drive age-related disease[1] (Figure 2C).

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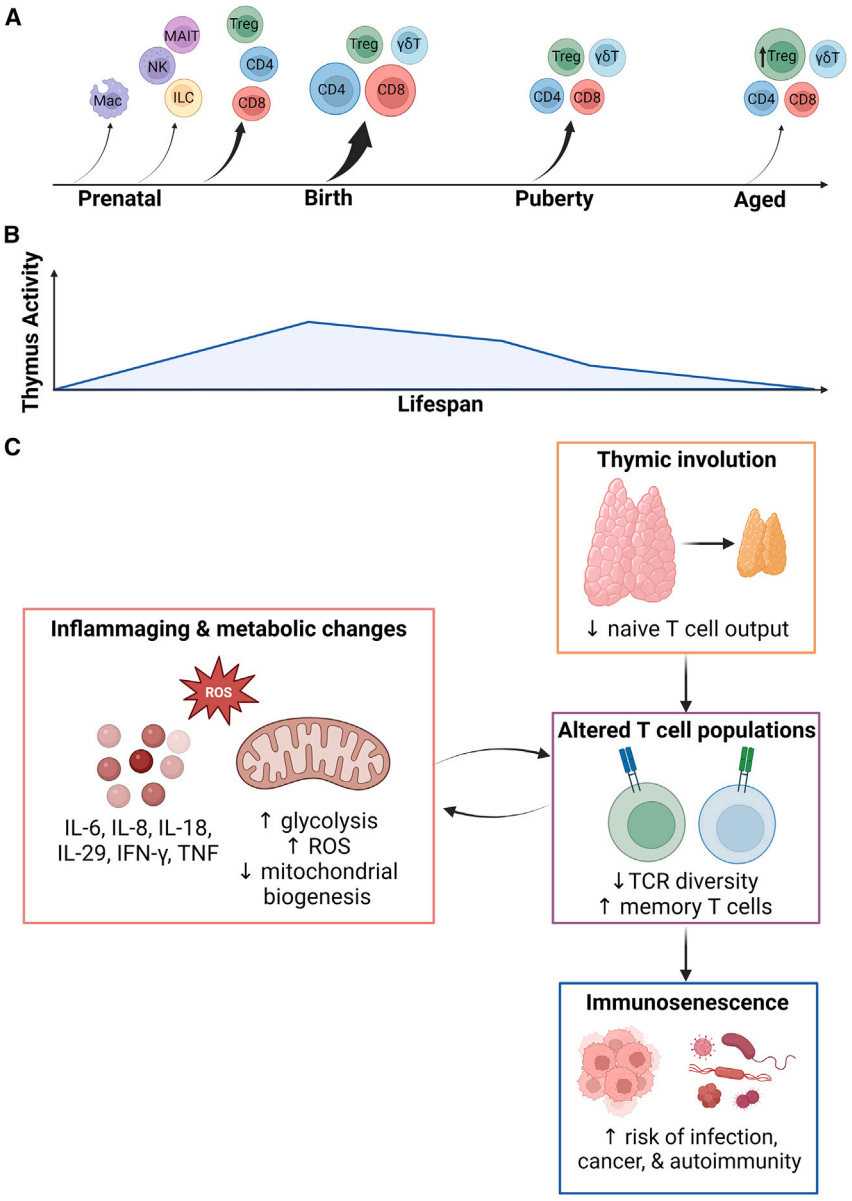

Figure 2. Thymic output changes and progressively declines with age, driving age-related immune impairment in the elderly

(A) Immune cell subset production changes throughout life, with increased innate-like phenotypes emerging during early fetal development and increased T regulatory cells in aged individuals.

(B) Thymus activity, defined by thymus size and productivity, changes drastically with age. The thymus grows rapidly during fetal development and the first year of life and then begins to decrease production. The decrease in function accelerates during puberty and continues to decline until thymic function and activity have been significantly reduced, generally occurring between 50–65 years of age.

(C) Thymic involution acts as an early driver of age-related immune impairment via decreased production and alterations in the output of specific T cell subsets. This results in decreased TCR diversity, which, combined with age-related defects in immune cells and increased basal levels of inflammation, lead to decreased immune function and drive age-related disease.

The initiating events: interplay on molecular, cellular, and physiological levels

Thymic involution is catalyzed by several mechanisms that regulate the progressive decline in T cell output and transition toward adipose tissue. For example, fetal thymus development and regulation of the size of the early thymic progenitor (ETP) niche have been shown to be in part regulated by ETPs migrating from the fetal liver or bone marrow.[46] In early fetal development, these cells provide necessary cues to support thymic epithelial cell (TEC) differentiation into cortical and medullary TECs, a step that is necessary to generate the spatially defined architecture of the thymus lobule that supports positive and negative selection of thymocytes. Throughout life, ETPs continue to provide essential crosstalk signals with TECs, as exemplified by the ability of young progenitor T cells injected into the bloodstream to migrate to the involuted thymus and regenerate the developmental niche.[47,48] However, with age, there is a decrease in HSC proliferation and release of ETPs from the bone marrow, leading to a decrease in progenitor T cell-TEC crosstalk signals.[48–50]

The thymus is also responsive to hormonal changes, which have been shown to affect the stromal compartment and initiate changes in thymic architecture.[51] For example, thymus regeneration has been observed following castration.[52] The expansion of thymic stromal cells is catalyzed by a lack of androgen signaling, as demonstrated by studies in mouse where androgen blocker treatment stimulated an increase in *FOXN1* expression in TECs, driving their proliferation, thereby decreasing the rate of thymic involution and increasing the rate of rejuvenation.[51,53–57] However, the regenerative effect is temporary, with the thymus returning to pre-castration size within 1–2 months.[58] Similarly, the thymus is responsive to estrogens and progesterone, where it undergoes rapid involution during pregnancy and then rebounds to its original size post-birth.[59,60] Growth hormones, thymopoietin, glucocorticoids, and other signaling pathways have also been shown to control thymic size, activity, and regeneration, some of which have been tested in clinical trials to increase thymus activity in age-related disease or support immune system regeneration with HIV treatment.[51,61,62] These hormonal thymic rejuvenation studies have shown promise in increasing T cell generation. However, there have been serious off-target complications due to the pleotropic nature of these hormonal signaling pathways that have limited clinical therapeutic adoption.

Furthermore, when discussing strategies for thymus regeneration, it is important to address the reversibility of this process and rejuvenation potential of this organ. For example, under natural stressors, such as during puberty, pregnancy, castration, or

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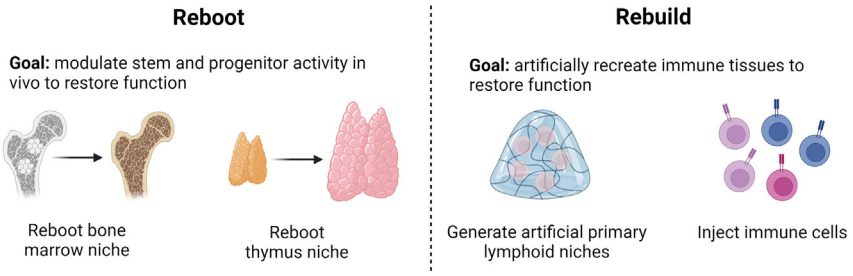

Figure 3. The two approaches to regenerating aged and damaged immune organs include rebooting the patient's own immune system and rebuilding the patient's immune system through artificial supplementation

To reboot the patient's immune system, current technologies aim to provide cells or drugs that regenerate the proliferative capacity of HSCs in the bone marrow or regenerate the T cell developmental niche in the thymus to improve the production of naive T cells. To rebuild the patient's immune system, technologies aim to generate artificial primary lymphoid niches that support immune cell development *in vivo* or *in vitro*, as well as direct supplementation approaches that produce T cells *in vitro* for direct transplantation into a patient.

infection, thymus size and function can rapidly decrease and then regenerate to steady-state function once the stressor has been removed or homeostasis restored.[34–37,52,59,60] This seemingly inherent property of the tissue to respond rapidly to the need for progenitor T cell niche availability and then return to steady-state function has also been demonstrated using artificial thymus regeneration strategies discussed below.[47,48,63,64] This regenerative capacity of the thymus argues that even in aged individuals, thymus tissue has the potential to regenerate functional niches to produce naive T cells.

Sex differences are prevalent in age-related immune impairment

More recently, sex differences in immune system reactivity and differential aging kinetics have come to light.[32,40,65–67] In 2001, comparison of signal joint TCR rearrangement excision circles (sjTRECs) in peripheral blood, a marker used to estimate thymic function via the number of recent thymic emigrants, established that females contained significantly higher levels of sjTRECs from age 20 onward.[32] This demonstrated that females have increased thymic output of naive T cells throughout middle and later life, which would support greater TCR diversity and ability to respond to new pathogen challenges with age. These data were further supported by a recent study on peripheral blood from 172 healthy adults, which characterized sex differences in the age-related kinetics of declining naive T cells and increasing monocyte and cytotoxic cell functions. Two spikes in the rate of these detrimental changes were detected, with the second spike occurring earlier and with a greater magnitude in males.[67] Additionally, after age 65, males had higher innate and proinflammatory activity, as well as lower adaptive activity.[67] A separate study demonstrated higher T cell activation signals in young females and again documented sex-specific differences in the composition of immune cells and levels of inflammatory factors with age.[66] These changes in the peripheral immune system provide an accessible, if delayed, method of assessing declines in thymus contribution to immunity and generally mirror sex differences in thymus activity and involution throughout life.

In the context of the thymus, sex hormone regulation of thymic involution has long sparked a large body of evidence documenting sex differences in thymus biology, involution, and T cell generation.[57,68] Decreased production of recent thymic emigrants and smaller overall thymus size in older males versus females have also been reported.[32,69] With respect to cell and niche-level changes, some studies describe decreased numbers of AIRE$^+$ mTECs with age, potentially predisposing females who maintain greater thymic function later in life to autoimmune disease,[53] as well as less interlobular fat in young female thymus,[69] suggesting that differences in thymic involution kinetics begin pre-puberty. Recently, we described how transcript-level sex differences may underlie functional differences in thymic and immune function in humans.[29] Our analysis of thymic cell gene expression uncovered differential regulation of pathways involved in cell-cell signaling and metabolism, with female-derived thymic cells having significant upregulation of metabolic, translation, and antigen presentation pathways, whereas male thymic cells had increased adipogenesis, proinflammatory signaling, and glucocorticoid signaling.[29] Importantly, these differences were found in 4–5 month-old infants, demonstrating that sex differences in immune system development begin pre-puberty and have the potential to differentially affect the process of T cell development and T cell reactivity in the periphery via differential regulation of negative selection in the thymic medulla. We hope to highlight these and other patient population-specific considerations as we discuss the state of therapeutic strategies to date.

INTERVENTIONAL STRATEGIES TO REBOOT AND REBUILD IMMUNITY

At present, there exists two schools of thought for interventional immune regeneration strategies in preclinical development: those that focus on regenerating aged immune organs to reboot a patient's immune system and those that focus on rebuilding the immune compartment directly through artificial supplementation of cells, niches, or tissues (Figure 3).

Technologies to reboot the immune system

Strategies to reboot the immune system focus on developing drugs and precision medicine technologies that push the native immune system back to a productive, healthy state. Rebooting the immune system via cell transplantation has a long history of support stemming from the insights of parabiosis experiments and successes of bone marrow stem cell transplants, as well as more recent work with chimeric antigen receptor (CAR) T cell therapies.[70–72] Current strategies include HSC or progenitor T (pro-T) cell transplants, which rejuvenate the proliferative capacity of the bone marrow and thymus, respectively, as well as drug strategies targeting the regenerative capacity of progenitor cells in the bone marrow and thymus (Table 1).

HSC transplants

In the clinic, bone marrow transplants act as life-saving therapies where they can regenerate a full immune system in cancer patients following chemotherapy and radiation.[72] For in-depth

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Table 1. Summary of the current technologies to reboot the immune system by pushing the native immune system back to a productive, healthy state

Technology	Description	Advantages	Disadvantages	References
HSC transplants	allogeneic transplant of bone marrow grafts containing HSCs to reconstitute the hematopoietic system	• long history of successful clinical data • can reboot the potential of all hematopoietic lineages (myeloid, megakaryocyte, lymphoid, etc.)	• adult donor cells may have genetic and epigenetic defects that limit therapeutic potential • limited donor availability • risk of GVHD • requires severe conditioning regime pre-therapy	Khaddour etal.,[72] Bacigalupo et al.,[73] and Peffault de Latour et al.[74]
PSC-derived HSPC transplants	transplant of *in vitro* PSC-derived HSPCs to reconstitute the hematopoietic system	Allogeneic • no donor required • potential for low-cost, large-scale manufacturing Autologous • potential for use of donor cells	• unknown engraftment potential • may require severe conditioning regimen pre-therapy • does not address limitations of aged bone marrow defects	Sturgeon et al.[75] and Taberna et al.[76]
Progenitor T cell transplants	transplant of progenitor T cells to rejuvenate the thymus microenvironment and increase naive T cell production	• improved recovery kinetics of T lymphoid compartment, especially in aged individuals • progenitor T cells temporarily regenerate the thymus • potential for low-cost, large-scale manufacturing	• temporary thymus reconstitution	Singh et al.[47] and Mohtashami et al.[48]
Bone marrow regeneration therapies	drug regime targeting bone marrow HSC activity and niche characteristics	• potential to reset epigenetic changes limiting proliferation and lymphoid skewing of aged HSCs	• limited clinical research to address defects in aged bone marrow niche or HSCs *in vivo*	Zeng et al.[77] and Montserrat-Vazquez et al.[78]
Hormonal regeneration therapies	drug regime of hormones, such as recombinant growth factor, known to increase thymus activity	• clinical results demonstrate increase thymus activity and naive T cell output in HIV-infected adults • clinical results demonstrate decreased epigenetic versus chronological age in older men	• mechanism of action is not well characterized • pleiotropic effects of hormonal drug regimes increase risk of off-target consequences	Napolitano et al.,[61] Fahy et al.,[62] and Napolitano et al.[79]
FOXN1-driven therapies	drug regime of small molecules that drive FOXN1 expression in TECs to increase thymus activity	• well characterized, driven by TEC differentiation, proliferation, and function • increased FOXN1 expression in mouse increases thymus size and production of naive T cells	• small-molecule and epigenetic regulators of FOXN1 function remain elusive • FOXN1 may differentially regulate activity and function of different TEC subsets • delivery must be tissue specific	Nowell et al.,[80] Suet al.,[81] Vaidya et al.,[82] Chen et al.,[83] and Li et al.[84]
Mesenchymal cell-driven therapies	drug regime or cell therapy targeting mesenchymal cells regulates the progression of thymic involution to modulate thymus architecture and activity	• mesenchymal cells are known regulators of thymic involution; therefore, blocking mesenchymal cell-driven involution signaling pathways could prevent, slow, or reverse thymus involution	• cell signaling pathways regulating age-related thymic involution are unclear • optimal onset of treatment is undefined • challenging to deliver to specific tissues	Jenkinson et al.,[85] Gustafsson et al.,[86] and Tan et al.[87]

discussion on the current knowledge of bone marrow niche regulation of HSCs in health, disease, and aging, as well as the state of HSC preclinical and clinical work, we refer readers to the following reviews.[72,88–93] Despite the efficacy of HSC grafts, they exhibit important age-related declines, with grafts in aged patients failing significantly faster than those in young patients.[73,74] Similarly, studies using the cord-blood expansion molecule UM171 have found that the increase in numbers of naive T cells, recent thymic emigrants, and T cell clonotypes were higher in younger patients (<40 year old).[94] Additionally, the remaining HSC clones in elderly individuals often have accumulated a large burden of somatic mutations, which can have broad effects on their activity and function, including malignant proliferation, differential skewing toward specific hematopoietic lineages, and inflammatory activity leading to cardiovascular disease.[95–97] However, the cost, limited donor availability, and clinical risks associated with HSC transplants have limited their extension to age-related disease in elderly patients.

Limited donor availability and questions of age-related defects in HSCs have motivated several labs to develop protocols for

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generating PSC-derived hematopoietic stem and progenitor cells (HSPCs) with multi-lineage potential. If these cells can be generated at scale, as is being pursued as an alternative to patient-derived bone marrow transplants, they could provide an unlimited source of HSPCs that could reboot the hematopoietic system in patients with age-related defects, especially if transplant barriers and conditioning challenges can be overcome.[98] In 2001, Kaufman et al. first demonstrated evidence of blood colony-forming cells from human embryonic stem cells (ESCs).[99] Since this discovery, the field has pursued protocols to direct development toward definitive-wave HSPCs with the capacity to give rise to functional lymphoid cells. In 2014, Sturgeon et al. showed that activating Wnt signaling during differentiation generated $CD34^{+}CD43^{-}$ hematopoietic endothelium cells with T-lineage competence.[75] Current research has focused on scaling the production of these HSPCs with T lymphoid potential in bioreactor systems.[76] Protocol modifications to increase yield and engraftment potential have recently been reviewed here.[100–104] These technologies have greatly expanded our ability to generate PSC-derived HSPCs; however, only recently was serial transplantation demonstrated in mouse,[105] and the long-term engraftment potential of PSC-derived HSPCs remains to be demonstrated in human. Additionally, this approach addresses age-related cell-intrinsic defects, such as somatic mutation burden, but does not address age-related bone marrow niche changes, which prevent skewing of HSCs toward T-lineage and may continue to bias transplanted HSCs in aged bone marrow toward myeloid phenotypes. Furthermore, given the known propensity of PSC-derived cells to exhibit fetal-like transcriptional programs and functional properties,[106,107] there is currently an important focus on understanding how functional properties of HSCs produced along the developmental axis of the aorta-gonad mesonephros region of the early embryo, the fetal liver, or the postnatal bone marrow will impact immune competence.[40,108–110] Improved characterization of HSCs produced along this developmental axis,[111] including PSC-derived cells produced in systems mimicking these niches,[112] is required to understand how these cells will function after transplantation to create a competent host immune system. These limitations have encouraged development of alternative strategies to support T cell development in aged patients.

Progenitor T cell supplementation to reboot the thymus

Bone marrow transplants often also exhibit a significant delay in, or lack of, naive T cell generation in patients with damaged or non-functional thymic tissue, either due to patient age or the damaging effects of chemotherapy and radiation.[113–115] The delay or absence of T cells in patients who receive HSC transplants leads to several complications such as opportunistic infections and increased predisposition to relapse.[116] To address these complications, Reimann et al. generated human pro-T cells from cord blood in a feeder-free culture system containing immobilized Notch ligand delta like 4 (DLL4) and transplanted these cells into non-obese diabetic (NOD)/severe combined immunodeficiency (SCID)/$\gamma c^{-/-}$ (NSG) mice.[64] The pro-T cells migrated to the thymus and developed into functional, mature T cells, accelerating the timeline for peripheral T cell reconstitution. Similarly, Awong et al. generated cord-blood-derived human pro-T cells *in vitro* using a co-culture platform of OP9 mouse stromal cells expressing the Notch ligand delta like 1 (DLL1) and co-transplanted the pro-T cells with HSCs into NSG mouse recipients.[63] They also observed accelerated HSC-derived T-lymphopoiesis and identified a potential receptor activator of nuclear factor κb (RANK) ligand-driven mechanism of thymus tissue regeneration. Singh et al. harnessed this pro-T cell-TEC signaling axis to regenerate the functional capacity of aged thymus by engrafting *in vitro* co-culture-generated cord-blood-derived human pro-T cells into aged mouse thymuses post-irradiation.[47] The pro-T cells homed to the thymus and were found to reconstitute the peripheral T cell compartment significantly faster than HSC transplant controls.[47] Follow up on this work again co-administered HSC transplants with *in vitro* co-culture-generated cord-blood-derived human pro-T cells and showed the accelerated reconstitution of CD4 and CD8 T cells in the peripheral T cell compartment of aged mice lowered the risk of opportunistic infections.[48]

As a first clinical target, pro-T cell therapy, or even lymphoid skewed blood progenitors,[50] holds promise for improving immune recovery following irradiation and chemotherapy in patients. However, the strong response seen with transplanted pro-T cells, including an increased ability to respond to viral infections, suggests that this type of therapy could also be used to boost immune function in elderly patients who do not require an HSC transplant post-radiation. These successes set the stage for use of cell-based immune regeneration strategies beyond cancer therapy and necessitate careful consideration of how different pro-T cell generation technologies would enable treatments for different patient profiles, specifically aged patients who do not require chemotherapy and radiation.

Drug strategies for thymus regeneration

Current small-molecule and targeted therapeutic strategies focus on harnessing mechanisms of thymus regeneration, such as stimulating *in vivo* production of T-lineage skewed HSCs or blocking mechanisms of thymic involution by targeting thymic epithelial and mesenchymal cell activity.

Bone marrow-targeted strategies. An alternative to introducing pro-T cells into patients is to directly stimulate HSC proliferation and differentiation toward T-lineage-skewed thymic seeding progenitors, which express thymus-homing chemokines and T-lineage-specific transcription factors.[117] However, it remains unclear what specific changes or defects occur in aged HSCs to inherently limit T-lineage potential or if there are specific changes to the bone marrow environment that limit the proliferative and T-lineage skewing potential of aged HSCs.

To identify the specific molecular changes occurring in aged HSCs, Zeng et al. used multi-omic sequencing on a xenograft inflammation-recovery model and found a pool of inflammatory memory HSCs that accumulated with age and clonal hematopoiesis.[77] These cells exhibited a distinct transcriptomic and epigenomic signature that was enriched for inflammation response and long-term HSC quiescence.[77] These data require further functional studies to determine how this signature could affect HSC skewing toward myeloid lineages and quiescence versus T-lineages and proliferation, as well as if these cells are amenable to gene editing or epigenetic modification to reverse this inflammatory, dormant signature. To this end, one study used the small-molecule UM171 during *in vitro* cord-blood HSC expansion to increase T cell clone numbers and TCR diversity post-transplantation, demonstrating that small molecules

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could be used *ex vivo* to modulate the epigenetic state and activity of HSCs *in vivo*.[94]

Furthermore, a recent study by Montserrat-Vazquez et al. performed imaging of aged bone marrow and found that aged HSCs reside in a distinct niche from young HSCs.[78] These data suggest that age-related changes to the bone marrow niche, such as the distribution and density of Notch ligands, may limit further HSC proliferation. Currently, we lack technologies that directly modulate levels of regulatory ligands and signaling pathways, such as Notch signaling, within the bone marrow niche. Instead, these niche-level changes to HSC regulation have been addressed with artificial bone marrow niches,[50] as discussed further below.

Hormonal regeneration strategies. To date, clinical research on direct thymus rejuvenation has primarily focused on artificially regulating specific hormones. For example, the effects of growth hormone on regulating thymic activity were demonstrated in patients with HIV who began taking highly active anti-retroviral treatment (HAART).[61] Patients who started receiving HAART treatment were seen to exhibit immune reconstitution with an increase in naive CD4 T cells.[118] These results demonstrated that the thymus is still active in these patients and can continue to produce naive T cells once disease progression has reached an equilibrium. Immune reconstitution of CD4 cells was then augmented via administration of growth hormone, which had been shown previously to result in thymus regrowth with an increase in thymus density, TREC frequency in peripheral blood, and number of total and naive CD4 and CD8 T cells.[61,79] In 2015, the Thymus Regeneration, Immunorestoration, and Insulin Mitigation (TRIIM) clinical trial began to investigate the possibility of using recombinant growth hormone to extend these results and prevent or reverse signs of age-related immune impairment in ten healthy 51–65-year-old men.[62] Although this study did report a decrease in epigenetic versus chronological age, growth hormone has additional known pro-aging effects, such as hypothalamic inflammation, which may program systemic aging in adults.[119,120] Regardless, the pleiotropic nature and concern for off-target effects of hormonal approaches have motivated precision medicine approaches to restoring thymus activity.

FOXN1-driven regeneration strategies. The main approach to rebooting *in vivo* thymic tissue function focuses on modulating *FOXN1* gene expression. *FOXN1* is essential for TEC differentiation and proliferation,[80–82] and premature downregulation of *FOXN1* in mice causes a rapid involution-like phenotype.[83] General overexpression of *FOXN1* in TECs has been shown in mouse to increase aged thymus size and thymopoiesis, as well as restore normal thymus architecture.[121] These results provided a promising target for clinical thymus regeneration. However, the mechanisms controlling transcriptional regulation of *FOXN1* remain unclear and an active area of research by academic and industry teams. Some teams are pursuing broad small-molecule screens of drugs to find chemical modulators of *FOXN1* activity, while other labs are taking a bottom-up approach by harnessing new multi-omic technologies to identify epigenetic regulators of *FOXN1* activity.[122]

As these strategies progress to the clinic, it is important to note a recent study in mice that overexpressed *FOXN1* in TECs using a *K5.Foxn1* transgene and drove *FOXN1* expression in *Plet1*$^{+}$ progenitor TEC cells.[84] Unlike other *FOXN1* overexpression models, this transgene did not increase thymus size or delay thymic involution but did improve TEC differentiation and was independently sufficient to drive formation of a small thymus in nude mice.[84] Importantly, these findings demonstrated the context- and dosage-dependent effects of perturbed *FOXN1* expression in different TEC subsets. Single-cell RNA sequencing studies from various groups have demonstrated surprising heterogeneity in functional TEC subsets,[123–128] including the presence of a thymic epithelial stem cell phenotype that expresses several keratin genes and can differentiate into both medullary and cortical TECs *in vivo* and *in vitro*.[127] The ratios of these diverse TEC subsets are dynamic throughout life, with studies in mice demonstrating a decrease in mTECs and increase in cTECs and progenitor TECs with age.[129] As *FOXN1*-driven strategies to rejuvenate thymus tissue mature, it becomes increasingly important to understand which subset of TECs a strategy aims to target and modulate.

Furthermore, as these strategies move toward rejuvenating aged thymus rather than thymus tissue damaged by HSC transplant conditioning regimens, it will be important to determine how the different ratios of TEC subsets, specifically an increase in the number of progenitor TECs in the elderly,[129] change the functional outcome of *FOXN1* overexpression. To address these considerations, we envision several different strategies maturing to the clinic concurrently as the indications for thymic tissue regeneration expand. For example, a small-molecule-driven general *FOXN1* approach might be successful in driving regeneration in young thymus tissue damaged by radiation, whereas a more targeted approach might be effective to drive regeneration in aged thymus. Regardless, expression of *FOXN1* in other tissues, such as the skin,[130] presents an open challenge to ensure delivery of this therapy is tissue specific.

Mesenchymal cell-targeted regeneration strategies

Finally, the strategies outlined above focus on countering thymic involution by driving TEC proliferation. These strategies do not address the driving factors of thymic involution, thought in part to be orchestrated by mesenchymal cells in the thymus.[85–87] In the postnatal thymus fibroblasts produce important growth factors to regulate TEC proliferation and maintenance, including fibroblast growth factors (FGF2, FGF7, and FGF10) and insulin growth factors (IGF1 and IGF2).[29,85,131] These mesenchymal-TEC interactions play a crucial role in thymus maintenance, as demonstrated by the congenital hypoplasia seen in patients with DiGeorge syndrome where mesenchymal cells fail to support thymic tissue expansion.[132,133] How these interactions change within the aging thymus is complex and not well understood. However, targeting mesenchymal cells—the orchestrators of thymic involution—presents an alternative and relatively untapped opportunity to prevent or delay thymic involution.

One recent study explored the role of thymic mesenchymal cells (ThyMCs) in thymic function and regeneration, demonstrating the potential of this alternative approach.[86] In representative mouse models of HSC transplant, aging, and chronic lymphoid progenitor deficiency, they found significant remodeling of the ThyMC compartment, specifically an increase in interstitial fibroblastic Penk+ ThyMCs and a reduction in perivascular T cell supportive Postn+ ThyMCs. The authors then tested if replenishing ThyMCs could enhance thymic regeneration in the context of HSC transplant and aging and transplanted

Penk+ and Postn+ ThyMCs or Ccl19-overexpressing bone marrow stromal cells in conditioned mouse models, respectively. In both conditions, stromal cell transfer resulted in improved short-term thymic regeneration via ETP recruitment, as well as long-term increases in peripheral naive T cells and improved vaccination responses. These data support ThyMC transplant as an additional strategy for thymus regeneration and also suggest that functional ThyMC modification via *in vivo* drug targeting, such as CCL19 overexpression, may pose an alternative, less invasive therapeutic strategy.

However, targeting ThyMCs as a therapeutic strategy requires further research in human and in the aged thymus niche to identify the signaling pathways dictating ThyMC activity and function. For example, DPP4+ mesenchymal cells have recently been characterized as having progenitor capacity, placing these cells at the top of the fibroadipogenic cell hierarchy in several tissues. DPP4+ ThyMCs have been identified in single-cell sequencing studies in mouse and human[29,86,134] and have the potential to differentiate toward fibroblast or adipogenic lineages. Without proper control of the complex signaling pathways guiding this lineage decision, as well as further differentiation toward the functional ThyMC subtypes described above, these therapeutic strategies risk irreversibly increasing thymus tissue fibrosis. As these strategies develop, it is important to account for key differences in mouse and human thymic involution pathways, namely a decreased volume of adipogenic thymus tissue in mouse,[135] as well as how emerging therapies may interact differently within an aged, fibrotic thymus environment.

Technologies to rebuild the immune system

Strategies to rebuild the immune system focus on technologies that artificially recreate immune tissues, either by creating an artificial niche for *in vivo* immune system generation or by direct injection of *in vitro*-derived immune tissue into the patient (Table 2).

Allogeneic thymus transplantation

The tissue-centric approach to rejuvenating T cell immunity focuses on rebuilding a thymic microenvironment to better support naive T cell generation. This approach has gained significant traction given the recent Food and Drug Administration (FDA) approval of thymus transplant studies.[154] The safety and efficacy of thymus transplants in children with congenital athymia were established in a clinical study that ran from 1993 to 2020, and the treatment is now established for this rare pediatric disease under the brand name *Rethymic*.[136–138] Rethymic uses allogenic thymus tissue that is processed, cultured, and implanted in a single administration, enabling generation of naive T cells and improving the survival rates of children with congenital athymia. This therapy represents a big step toward the establishment of thymic tissue transplantation as a feasible strategy to rebuild the immune system.

These successes suggest that thymus transplants, once established, could also be indicated in thymectomy and aged patients. For example, an intriguing new study demonstrated the continued importance of thymic function in aged individuals.[155] In this study, researchers compared a large cohort of patients who had undergone thymectomy with a primary control cohort and found that at 5 years post-surgery, all-cause mortality and cancer risk were higher in the thymectomy group. Thymectomy patients with no prior risk of infection, cancer, or autoimmunity also had an increased risk of autoimmune disease.[155] We believe these results could also be extended to demonstrate the importance of continued thymic function in the elderly. Past the age of 65, thymus production is significantly reduced,[31] catalyzing changes to the peripheral immune compartment consistent with thymectomy. It is likely that the success of thymus transplants in athymic children and literature on the continued importance of the thymus in adults will soon motivate thymus transplant and rejuvenation technology implementation in the elderly. However, the impact of the patient's residual circulating T cells on the probability of graft rejection is yet to be determined and will likely limit allogeneic transplants to patients who have already undergone radiation, as discussed below.

PSC-based strategies for thymus transplantation

The efficacy of thymus transplants has motivated PSC-derived thymus organ replacement strategies to increase therapeutic availability to a broader patient population. To this end, several groups are pursuing *in vitro* generation of PSC-derived thymic tissue, which can be implanted within the body to support naive T cell generation. This field was catalyzed by *in vitro* protocols that differentiated PSCs to TECs *in vitro*.[139–142] These approaches, which were recently reviewed here,[156] have been expanded in different model systems to push TEC differentiation toward mature, functional subsets of TECs. However, to support T cell development, these model systems require formation of three-dimensional (3D) spatially compartmentalized PSC-derived thymus tissue resembling cortical and medullary regions, which current technologies fail to recapitulate.

Currently, independent cultures of PSC-derived TEC cells resemble progenitor TEC or cortical TECs and likely require T cell and mesenchymal interactions to push these cells toward a medullary TEC phenotype, as seen in fetal thymus development.[157–160] One approach to this problem is to implant progenitor TECs under the kidney capsule and allow maturation by T cell-TEC interactions *in vivo*.[143] In this system, human PSCs are differentiated in 3D alginate capsules toward thymic epithelial progenitor cell (TEPC) lineage and co-cultured with cord-blood-derived HSPCs or cord-blood-derived pro-T cells in decellularized mouse thymic scaffolds.[143] The *in vitro* emigrant population of these thymic organoids co-cultured with pro-T cells contained both CD4 and CD8 single-positive T cells after 1 week of culture, and organoids cultured with HSPCs generated both mature populations of T cells within 2 weeks *in vitro*. When transplanted *in vivo*, peripheral lymphoid cells were detected within 4 weeks, with a prominent population of mature, TCR diverse, functional T cells detectable within 18–24 weeks. Finally, another group has demonstrated generation of an ectopic thymus under the kidney capsule from TECs induced from *FOXN1*-overexpressing embryonic fibroblasts.[144] Using *FOXN1*-reprogrammed embryonic fibroblasts injected directly into the aged murine thymus the authors found substantial regrowth of native aged murine thymus. These animals exhibited increased naive T cell generation, reduced senescent T cells in the periphery, and reduced T cell-mediated inflammation. These grafts provide an effective environment to support T cell development; however, similar to allogeneic thymus transplant, the probability of graft rejection due to residual patient T cells is yet to be determined.

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Hydrogel-based artificial primary lymphoid tissue niches

Complications arising from the delay or absence of T cells in patients who receive HSC transplants, as well as the limitations of introducing human leukocyte antigen (HLA)-mismatched PSC-derived thymus tissue into immune-competent individuals, have motivated technology development to create an artificial niche to support T-lineage skewing and pro-T cell development.

Bone marrow niche hydrogels. Age-related changes to the bone marrow environment may be limiting HSC activity and T-lineage skewing.[78] One approach to increasing HSC transplant engraftment and seeding of pro-T cells in the thymus is to use biomaterials to recreate the bone marrow niche to support and skew HSCs toward T-lineage. Biomaterial-based scaffolds do present several advantages, particularly for mitigating graft versus host disease (GVHD) in patients, as they rely on the host's own cells to seed the graft and facilitate T-lineage skewing. For example, Shah et al. developed an injectable bone marrow-like scaffold that releases bone morphogenic protein (BMP2) to recruit stromal cells and presents the Notch ligand DLL4 to facilitate T-lineage specification.[50] The graft was administered subcutaneously in mice and successfully increased T cell neogenesis and pro-T cell seeding in the thymus, improving survival post-HSC transplant. It will be interesting to see how effective this approach can be in elderly patients or patients who have undergone radiation and chemotherapy and have a damaged thymus environment. However, successes in pro-T cell supplementation therapies suggest that the pro-T cell-TEC crosstalk may be sufficient to rejuvenate the damaged thymus.[48]

Thymus niche hydrogels. Over the past two decades, thymic niche hydrogels have also been developed, which aim to recapitulate the three-dimensional (3D) thymus signaling environment to support T cell development. For example, a 3D inverted colloidal crystal (ICC) scaffold functionalized with the Notch signaling ligand delta like 1 (DLL1) was designed to act as a substitute for the structure and function of thymic stroma.[145] This group hypothesized that the intensity and configuration of cell-cell contacts are essential components to the function of thymic-like niches. This technology supported maintenance of cord-blood HSCs but did not support differentiation toward $CD4^+CD8^+$ double-positive T cells and did not allow easy recovery of the cells from the matrix for further characterization. In 2019, Kratzer et al. reported a polyethylene glycol (PEG) hydrogel functionalized with the Notch ligand DLL1 and the adhesion-supporting motif arginyl-glycyl-aspartic acid (RGD).[146] The authors report the generation of $CD4^+CD8^+$ double-positive T cells; however, the frequency of these cells generated in the hydrogel was less than 1% and was not significantly higher than plate-bound DLL1 controls. Further optimization of these technologies is required to mimic additional key interactions and signaling pathways to support efficient development of progenitor and mature T cells. For example, using multi-factorial chemical signaling screens *in vitro* T cell manufacturing systems have identified and employed defined culture media conditions containing developmental stage-specific signaling factors to efficiently direct T cell development toward double-positive and CD8 T cells.[112,151] These approaches have defined a set of cytokines and chemokines that direct T cell development *in vitro* and should be employed in combination with hydrogels to mimic the native signaling environment of the thymus and improve T cell yield. However, the hydrogel may require multiple formulations patterned into medullary and cortical zones with different mechanical and biochemical signaling properties to recapitulate the spatially defined environmental cues of the thymus. Finally, the application of these hydrogels, whether intended as an *in vitro* T cell production method or *in vivo* supportive niche for thymocyte development, should be clarified early in design, as the requirements for recreation of stromal signals or ability for stromal cells to migrate into and remodel the hydrogel will significantly affect choice of material and functionalization.

Thymus organoids

Thymus organoid cultures present an alternative technology for T cell generation, as well as a scalable platform for modeling the contributions of specific thymic cell types toward T cell development and TCR repertoire development. One platform, termed the artificial thymic organoid (ATO), aggregates cord-blood- or PSC-derived HSPCs with mouse MS5 stromal cells expressing DLL4 into a 3D structure suspended on transwell inserts in medium.[147,148] This platform is a 3D extension of the traditional OP9-DLL4 T cell generation system[161] and supports T cell development within the aggregate structure to produce mature CD4 and CD8 T cells. This system is xenogeneic, which adds challenges to clinical translation of T cells generated within the ATOs. Notwithstanding, the limited scalable nature of ATOs still enables its use in drug or gene mutation studies. For example, ATOs have been used to study T cell development from $CD34^+$ cells of patients carrying intrinsic hematopoietic or thymic defects that cause T cell lymphopenia.[149] This study demonstrates an important application of organoid technology, where age-related defects in HSCs could be screened to determine their effect on T cell development and potentially predict efficacy of different therapeutic immune regeneration strategies to guide treatment of specific patient populations.

In addition to the HSPC-seeded PSC-derived thymic organoids developed by Zeleniak et al. for *in vivo* transplantation described above,[143] complex thymic organoid systems have been designed for *in vitro* T cell production. Here, human PSC-derived thymic cell types such as mesenchymal and hematopoietic progenitor cells are also included in the aggregate to drive terminal differentiation of the TECs.[150] The combination of thymic cell types drove terminal differentiation of TEPCs *in vitro*, including expression of autoimmune regulator (AIRE) and tissue-restricted antigen presentation, but differentiation of mimetic TECs has yet to be shown. Importantly, these isogenic human PSC-derived organoids support mature T cell development *in vitro*, although CD4 development is limited.

These systems present an opportunity to probe human diversity in T cell development, where patient-derived organoids could be used to model the developmental impact of genetic defects in T cells or stromal cells or be used to screen potential therapeutic immune regeneration interventions on organoids derived from diverse patient populations. To achieve these goals, we require a better understanding of how each cell type contributes to T cell development, TCR repertoire formation, and thymus growth. Using well-defined input cell types and longitudinal characterization of organoid formation, isogenic PSC-derived thymic organoids present a useful model system to begin exploring these questions. For example, the signaling

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Table 2. Summary of the current technologies to rebuild the immune system using artificial primary immune tissues or tissue niches

Technology	Description	Advantages	Disadvantages	Reference
Allogeneic thymus transplant	donor thymus tissue is cultured and then implanted into the quadricep muscle in patients with athymia to provide a niche for T cell generation	• clinical success in patients with complete DiGeorge syndrome to reconstitute the peripheral T cell compartment and improve survival from infections	• success requires a lack of native T cells in patients • limited donor availability • increased risk of autoimmune disease • risk of GVHD in early treatment regimen • risk of TECs positively selecting T cells that do not fully match the host HLA	Markert et al.[136–138]
PSC-derived thymus transplant	PSC-derived thymus tissue is implanted to provide a niche for T cell generation	• increased tissue availability to patients	• success requires a lack of naive T cells in patients • risk of GVHD	Lai et al,[139] Inami et al.,[140] Parent et al.,[141] Sun et al,[142] Zeleniak et al.,[143] and Oh et al.[144]
Bone marrow niche hydrogels	DLL4-functionalized hydrogel is implanted subcutaneously post-HSC transplantation to provide a niche for HSC skewing toward T-lineage progenitors and increase of T cell generation kinetics	• no risk of GVHD • increases kinetics of peripheral T cell compartment regeneration	• requires functional, proliferative HSCs	Shah et al.[50]
Thymus niche hydrogels	functionalized hydrogels are seeded with HSPCs and cultured in media to provide a niche for T cell development	• no risk of GVHD • could be used as an *in vivo* thymus niche in patients with athymia or involuted thymus. • recapitulates the mechanical signaling environment of the thymus	• current technologies have low yield of progenitor or mature T cells *in vitro* • lack of *AIRE*+ cells increases the risk of autoimmune disease	Oh et al.[145] and Lee et al.[146]
Thymus organoids	key thymus cell types are combined and cultured with HSPCs to support T cell development	• present a technology to test perturbations to T cell and thymus development or screen patient characteristics • isogenic PSC organoids could be transplanted to support T cell production *in vivo*	• risk of GVHD • some xenogeneic models • lack of *AIRE*+ cells increases the risk of autoimmune disease	Zeleniak et al.,[143] Montel-Hagen et al.,[147] Seet et al.,[148] Bosticardo et al., [149] and Ramos et al.[150]
Defined manufacturing systems for PSC-derived T cells	defined media and culture conditions are optimized to support *in vitro* T cell generation in 2D and 3D systems	• reproducible—defined culture media, cell materials, and cell characterization • scalable-large-scale manufacture can decrease cost • cell product can be optimized for patient and disease (e.g., T_{reg}:CD4:CD8 ratio) • system can be used to screen genetic diseases or patient characteristics	• risk of GVHD • unknown TCR repertoire selection and compatibility with patients • inability to produce mature CD4 T cells	Iriguchi et al.,[36] Michaels et al.,[112] Shukla et al,[151] Suraiya et al.,[152] and Trotman-Grant et al.[153]

interactions directing TEC maturation, differentiation, and organization into structured thymus architecture during early fetal thymus development remain unclear. Although TEC maturation has been shown in current thymic organoid models,[143] it has yet to be shown if these models can generate the diversity of TEC subtypes required to recapitulate effective T cell selection *in vitro* or recapitulate the spatial organization of thymic architecture. If we can recapitulate spatial architecture, this system presents a unique opportunity to probe developmental thymus signaling networks by perturbing cytokine and chemokine signaling factors or blocking specific cell-cell signaling interactions.

Fully defined manufacturing systems

Although pro-T cell strategies hold promise for boosting the aging immune system, the cost and scale of this therapy as a preventative or age-related supplementation strategy are currently prohibitive. One approach for decreasing cost is to generate these pro-T cells from PSCs, therefore enabling scalable generation of an off-the-shelf product. For example, in our lab, we are interested in producing PSC-derived T cells in a chemically

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defined, feeder-free system.[112,151] This technology uses plate-bound DLL4 and the adhesion ligand vascular cell adhesion molecule 1 (VCAM1) in combination with a defined media of growth factors and cytokines to recreate the thymic niches T cells migrate through during development in the thymus. Similarly, other labs have used T cell-derived PSC cells to generate T cells *in vitro*.[36,162] This approach bypasses the need for recreating a niche for TCR rearrangement. However, under some conditions, these cells tend to express innate cell markers and could not be used to reconstitute a diverse T cell repertoire.[163–165] The lack of xenogeneic cells or byproducts is an inherent advantage of these platforms; however, scaling production of these cells has proven difficult due to the necessity of mechanical pulling forces to engage the Notch ligand DLL4.[166]

Recently, a method was developed to enable large-scale T cell production in a bioreactor using gelatin-based microgels encapsulating murine HSCs with OP9-DLL4 stromal cells.[152] This approach is an important step in generating scalable technologies for T cell development, but it did not demonstrate mature T cell production and could have limited clinical applicability due to its xenogeneic nature. An alternative non-xenogeneic approach uses DLL4-coated microbeads in combination with sequential lymphopoietic cytokines to differentiate cord blood, peripheral blood, and PSCs to CD3+ TCR$\alpha\beta$ mature double-positive T cells in a bioreactor system.[153,167] Similar to many feeder-free differentiation systems, progression of PSC-derived thymocytes toward mature single-positive T cell stages was limited, in part due to a lack of artificial stimulation of double-positive T cells with anti-TCR/CD3 antibodies.[153] This inability to differentiate PSC-derived cells toward mature CD4 T cells highlights a lack of understanding on how to guide T-lineage branching and recapitulate additional thymic medulla-like maturation signals in feeder-free or 3D bioreactor systems.

As these chemically defined manufacturing technologies mature to enable the generation of naive CD4, CD8, and T regulatory cells, they could allow targeted or population-level modeling of how T cell activation and function vary in different inflammatory environments. For example, *in vitro* functional cell assays recapitulating patient stratification axes such as age, sex, and blood biomarker inflammatory states via differentiation of diverse PSC lines in combination with disease-relevant culture media formulations can be employed to model patient-specific T cell responses. Establishing these *in vitro* screening models can assist in prediction of dose, effective T cell subset ratios, and patient responses to therapy.

CONSEQUENCES OF APPLYING IMMUNE REGENERATION STRATEGIES IN THE ELDERLY

Given the promise of these emerging technologies and the movement of several strategies toward the clinic to address age-related changes to the immune system, it is important to reflect on the evolutionary factors driving thymus involution. We and others are particularly interested in understanding why thymus involution initiates so early in human development and how the consequences of this phenomenon on human health may be exasperated in the context of aging and disease, further motivating immune therapies in the elderly. The "Wear and Tear" theory of thymic involution suggests that the breakdown of thymic tissue function is due to intense use early in life, resulting in deterioration of thymic cellular machinery.[168,169] However, this theory does not account for the fact that the thymus can robustly regenerate after periods of stress. The "Direct Adaptation" theory hypothesizes that, since the thymus is highly proliferative, thymus function must be decreased quickly to prevent thymomas and decrease the risk of autoimmunity.[170] Given this logic, the question remains why we don't observe a parallel decline in B cell production—a similarly proliferative tissue—and see an increased risk of autoimmune disease following removal of the thymus.[155] Finally, the "Disposable Soma" theory argues that organisms must optimize their investment in the maintenance of thymic tissue, where energy is invested early in life to produce a diverse pool of long-lived peripheral T cells, and T cell production is subsequently decreased to divert energy and resources toward the production of progeny and passing of genes to future generations.[171–173] Essentially, this theory claims that the process of somatic cell aging has evolved within the natural environment such that an animal will die of a non-age-related illness prior to loss of organ function becoming a sufficiently serious threat to life. For further commentary on the evolutionary cost-benefit analysis of continual production of different immune cells, the authors recommend the important commentary by George and Ritter.[33]

When critiquing this breakdown in immune function, it is important to consider that thymic involution was optimized and selected for in individuals who were not subject to advances in modern medicine and infrastructure that extend the lifespan of the elderly. Moreover, these individuals did not mature in our globalized society where pathogens can travel rapidly between continents and people live in highly populated cities burdened by pollution and increased probability of disease reinfection.[174] For example, thymic involution was optimized in individuals living in small hunter-gatherer groups with relatively little outside exposure to other groups or environments. Therefore, within the first few years of life, individuals would have been exposed to most pathogens within their environment.[33] Compared with today, the breadth of new pathogens and, importantly, the likelihood of repeat, immune polarizing exposures these individuals would be exposed to later in life pales in comparison to the number of repeat exposures individuals living in our globalized society are exposed to.[174] How does this rapid change in our environmental exposures affect how our peripheral T cell pool skews and remodels during our lifespan? These rapid societal changes argue that increasing the production of naive T cells in the elderly would increase TCR diversity, enabling better vaccination responses and response to new pathogens to potentially counteract this effect of globalization.

For these reasons, the Disposable Soma theory has gained traction and provided the groundwork to argue for continued production of T cells in the elderly. However, critics of these approaches continue to raise the important question of how artificial manipulation of the delicate balance of immunity could result in unintended autoimmune consequences. For example, we still do not have good models to understand how new naive T cells might react in an aged, inflammatory environment. In this argument, the authors find it helpful to view possible therapeutic perturbations along an autoimmunity-infection risk spectrum (Figure 4). If we consider T cell reactivity along this spectrum, with aged patients having decreased immune

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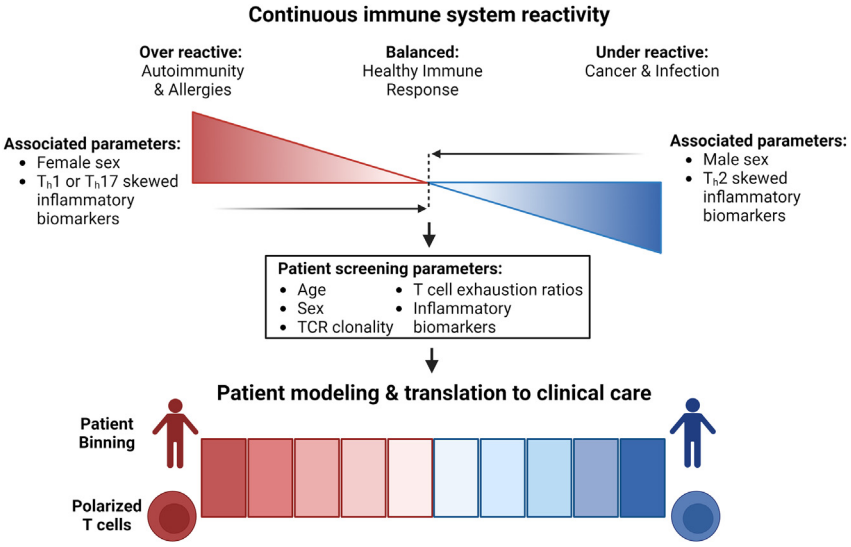

Figure 4. The autoimmunity-infection risk spectrum

A healthy immune response requires a regulated response of T cell activation against pathogens and mutated host cells, which is contained to limit damage to surrounding tissues. An under-reactive response, which has been associated with individuals of male sex and T_h2 skewed biomarkers, lacks the ability to clear infections and cancerous tissue, whereas an over-reactive response, which has been associated with individuals of female sex and T_h1 or T_h17 biomarkers, can result in autoimmune or allergic disease. Immune regeneration therapies aim to push the balance of immunity toward a healthy immune response. To safely achieve this the initial state of the patient's immune system along this spectrum must be well characterized, and the effect of artificially altering the patient's immune system must be defined. This could be done by collecting patient biomarkers of age-related immune impairment and inflammaging as screening parameters and binning patients accordingly along this spectrum. Patient responses to a given therapy could be modeled for each bin by recreating the inflammatory immune environment with bin-specific culture media and T cell state conditions.

reactivity, our goal with immune supplementation is to push the under-reactive immune response back toward the center of the scale. However, this push needs to be tightly controlled, as the unknown consequences of tipping the scales toward autoimmunity could be equally severe. It is also critical to consider that this scale is likely non-linear and personalized to each individual's immunological fingerprint, which includes axes such as age, sex, TCR clonality, T cell exhaustion ratios, and levels of inflammatory biomarkers. This is not said to discourage progress on countering age-related immune impairment, but instead to argue for concurrent modeling of patient populations and immune system reactivity as regenerative therapies develop. For example, we could imagine binning patients based on biomarkers of inflammaging and age-related immune impairment and using this stratification to determine dose and applicability of different therapies. Our goal here is to pose the question of risk-reward of immune regeneration and spark discussion on how to model the extreme heterogeneity seen in immune responses in the elderly. The benefits of immune regeneration are vast and exciting, but the consequences are highly relevant to long-term health. We hope to highlight how emerging technologies can be used to model patient immune systems and therapeutic responses to address these conflicting perspectives in our discussion below.

NEXT STEPS AND FUTURE PERSPECTIVES

Given the incredible progress the field has made toward rejuvenating the immune compartment, it has become imperative to revisit questions of patient applicability, timing, dose, and control of our artificial manipulations. How do we account for known differences in immune responses—not only accounting for age but also accounting for sex, race, and environmental stresses? What platforms for drug testing are we currently lacking to empirically answer these questions? Here, we discuss the potential of emerging technologies to address these questions via *in vitro* and *in silico* modeling (Figure 5).

Modeling HLA mismatching and determining drivers of tolerance

Surprisingly, thymus transplants do not require HLA matching to prevent GVHD, and preliminary results have even shown an increase in the number of CD4 T cells produced in complete HLA-mismatched grafts versus grafts with partial HLA matching.[136–138] These results are surprising, as one would assume the TECs contained within the grafts would tolerize T cells to graft HLAs and not to host HLAs, resulting in naive T cells released to the periphery that are reactive to host tissue. It has been suggested that the observed tolerance in these patients may be due to infiltration of hematopoietic cells into the tissue,[138] either as mature dendritic cells or via differentiation of ETPs toward alternative lineages such as dendritic cells.[175–181] Stromal cells may also migrate into thymic tissue to form the vasculature or to encapsulate the transplanted tissue. Thymic fibroblasts have been recently shown to play a role in medullary selection and tolerance[134] and possibly in the selection of cortical thymocytes as well.[29] Host epithelial cells could also migrate into the graft, as is seen in lung transplants.[182,183] However, the presence of these host cells within the engrafted tissue has only been hypothesized and not definitively shown, as almost all the patients who survived the first year post-thymus transplantation were alive at the culmination of the clinical study.[137]

Given the importance of thymic tolerance mechanisms such as TEC expression of *AIRE* and forebrain embryonic zinc (FEZ) family zinc finger 2 (*FEZF2*), or the presence of mimetic cells in the medulla,[184] the success of these grafts is still remarkable. DiGeorge syndrome patients who have received these transplants have manifested autoimmune conditions[136,137]; however, it remains unclear if the increased risk of autoimmune disease is due to the gaps in thymic tolerance created by the lack of TEC tolerance mechanisms or if it is due to alternative mechanisms associated with DiGeorge syndrome, which generally presents with increased risk of autoimmune conditions.[185] Regardless, these results raise important questions that need to be addressed if PSC-based allogeneic strategies for thymic

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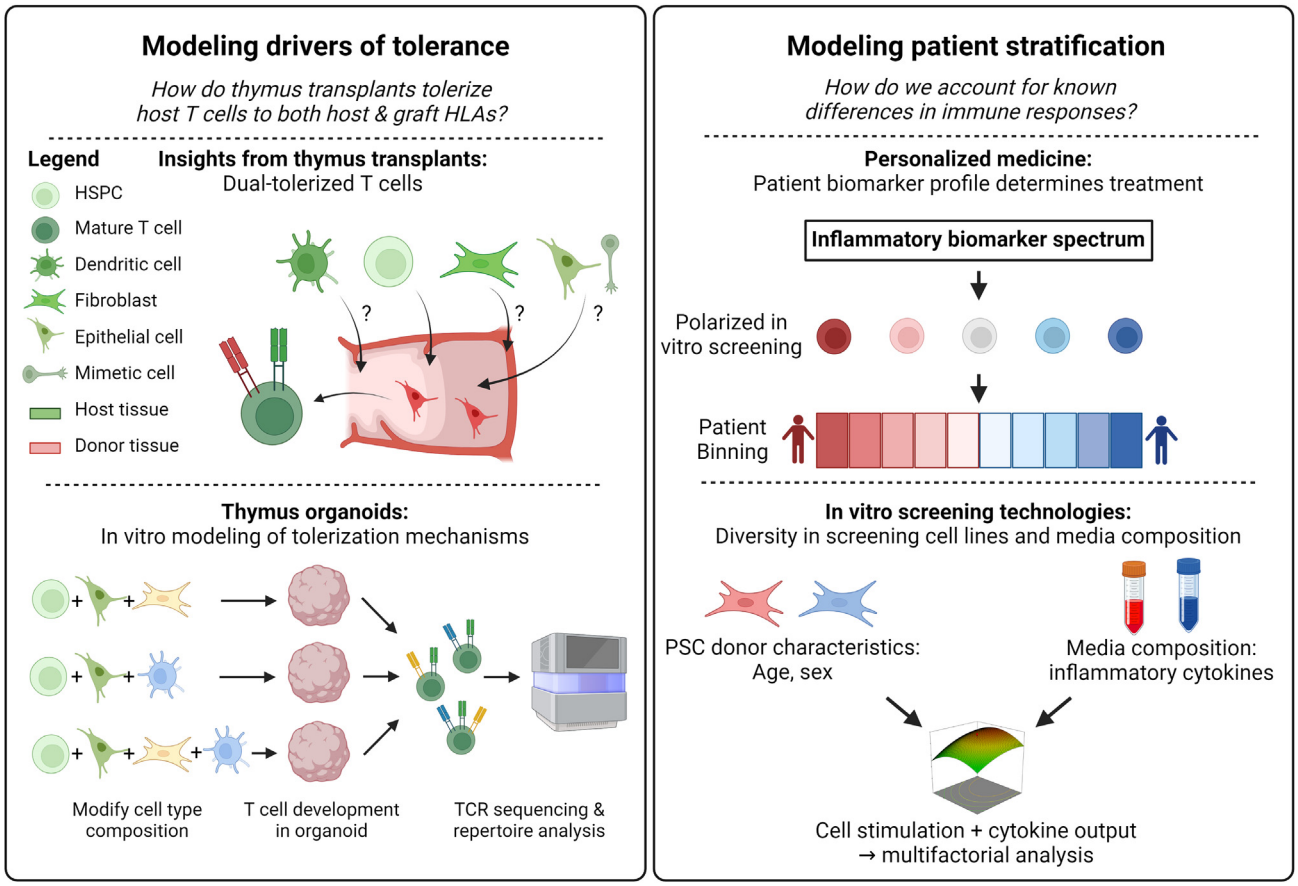

Figure 5. Continued progress of emerging immune regeneration therapies requires improved understanding of how transplanted cells and tissue interact and function with the host immune system, as well as technology development for *in vitro* modeling of diverse immune system states

Key insights into the drivers of tolerance emerged from thymus transplant studies where new T cells are tolerized to both host and donor HLA profiles. Further progress to unlock the potential of allogeneic, hydrogel-based, and PSC-based thymus tissue transplants requires improved understanding of how different thymus cell types contribute to TCR repertoire formation and selection. Modeling of patient stratification characteristics is required to enable safe and personalized immune regeneration treatment. Ideally, therapies would be screened in conditions representative of the patient's immune system to predict how the immune system will respond. To achieve this goal, we need to incorporate and characterize diversity in PSC donor cell lines and culture media conditions to generate predictive models of the function of emerging immune therapies in different patient populations.

transplantation are to be pursued to increase availability of these therapies and expand possible indications.

Providing further insight, children who have received thymic transplant grafts thus far were athymic and had $<10^6$ cells/L circulating naive T cells.[138] This lack of T cells, along with a course of immunosuppressants during the first few months after thymus transplant, has resulted in minimal complications where host immune cells attack the HLA-mismatched graft.[136–138] These clinical results are encouraging as it is possible that patients who receive PSC-derived thymic grafts may not require lifelong immunosuppression, the effects of which may counter many of the benefits of the enhanced T cell compartment. However, it remains to be seen if patients without complete DiGeorge syndrome will require myeloablative therapy to ablate the host immune system and if the levels of T cells remaining in the patient post-myeloablative therapy would be sufficient to prevent graft rejection complications.

Furthermore, the autoimmune complications seen thus far necessitate further investigation of the TCR repertoire changes these grafts produce. PSC-derived thymic organoid models present a unique opportunity to test questions regarding which thymic cell subsets contribute to the development and tolerance of specific TCR clones and how alterations to the ratios of different thymic cell types can skew the TCR repertoire. Current thymic organoid systems can combine PSC-derived TECs, fibroblasts, hematopoietic cells, and pro-T cells.[150] One could design an experiment where each cell type in a thymic organoid has a specific HLA identity and incorporate these cells in different cell type combinations. The pro-T cells would develop in environments where they are tolerized to HLAs by specific cell types, and the resulting TCR clonal diversity could be read out via single-cell TCR sequencing to determine how specific cell types shape TCR repertoire formation. Insights from these *in vitro* models, which can be performed in higher-throughput, controlled experiments, would provide important insights regarding the impact of non-epithelial cells on TCR repertoire selection in the thymus.[134,186]

Modeling patient stratification and the impact of the aged immune system

An emerging question in the field is how patient attributes should affect the choice of strategy, timing, and applicability of a therapy. Current research from our labs has emphasized the presence of sex differences in thymus biology and thymic involution kinetics.[29] We provided evidence of thymus sex differences in 4–5 month-old infants, capturing both common sex differences seen in other body tissues that affect cell metabolism and reactivity to stimulation, as well as thymus-specific differences in the training environment of T cells. Furthermore, we found evidence of differential thymic involution kinetics between sexes, with male cells demonstrating increased expression of adipogenesis pathways even at this early developmental stage. This research contributes to a large body of evidence that thymic involution kinetics differ significantly between sexes and, given the strikingly early onset of thymic involution, highlights the important question of when therapeutic strategies should begin. Should tissue-centric thymus rejuvenation strategies begin early in life to prevent thymic involution? Or should they aim to restore function in middle-aged individuals to extend thymus productivity? We would argue that research on young-to-old blood transfusions demonstrates that early intervention before thymus involution begins could be advantageous, given the known damaging influence of inflammatory environmental factors in aged tissue on progenitor cell function.[70] However, the timeline for readouts for these preventative therapies would be long and difficult to justify, and the thymus possesses the capacity to regenerate even after involution has begun.[47,48] Additionally, there is a concern for increased risk of autoimmune disease with increased naive T cell generation in middle-aged individuals. Further research on the impact of countering thymus involution in otherwise healthy adults and clarification of the target patient populations and timing of the implementation of emerging thymus rejuvenation technologies are required.

It is also prudent to consider the level of precision and control we require for these therapies when we assess therapeutic design strategies. For example, if employing a cell supplementation approach from defined manufacturing systems, do we need to have defined ratios of different T cell subsets? In CAR T cell therapy, inclusion of both CD4 and CD8 CAR T cells shapes the overall expansion kinetics of therapeutic products and is crucial for maintaining long-term responses.[187] To precisely engineer the immune system in the context of age, it will be essential to understand the contribution of individual T cell subset ratios in modulating immunity and how *in vitro*-derived naive T cells will function in an aged, inflammatory environment. Similarly, if thymus function is rejuvenated under aged inflammatory conditions will the peripheral immune compartment reach a healthy homeostasis?

If technologies to reboot and rebuild the immune system are to be successful in preventing and treating age-related diseases, we need to have a clear understanding of how the aged systemic environment will affect cell function, as well as how the function of key cell types involved in TCR repertoire selection, such as *Aire*+ and mimetic cells, change with age. Again, we reference the autoimmunity-infection spectrum and argue that binning patients based on health history and biomarker status provides a first-attempt approach at empirically answering these questions. For example, in the case of cell supplementation approaches, *in vitro* human-on-a-chip models could be developed where culture media are tailored with differential cytokine concentrations to reflect the aged inflammatory environment spectrum. By changing the ratios of CD4, CD8, and T regulatory cells included in these models and measuring changes in T cell skewing and function, we can begin to develop *in silico* models of how cell therapy products can be tailored to different immune systems.

CONCLUSIONS

Overall, important unanswered questions remain regarding the feasibility and safety of immune regeneration approaches outside the context of HSC transplantation and genetic immune system defects. As strategies discussed herein progress toward the clinic, consideration of the implications in clinical use and design parameters, such as timing of application, interaction with the host immune system, and functional characterization of the target patient population, become increasingly important. We would argue that these parameters should be a primary consideration guiding design of new therapeutic strategies, with knowledge on the timing of implementation and target patient population guiding the choice of strategy.

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DECLARATION OF INTERESTS

P.W.Z. is a cofounder of Notch Therapeutics and a member of its scientific advisory board.

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