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Restoring thymic function: Then and now

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ABSTRACT

Thymic vulnerability, a leading cause of defective immunity, was discovered decades ago. To date, several strategies have been investigated to unveil any immunorestorative capacities they might confer. Studies exploiting castration, transplantation, adoptive cell therapies, hormones/growth factors, and cytokines have demonstrated enhanced *in vitro* and in *vivo* thymopoiesis, albeit with clinical restrictions. In this review, we will dissect the thymus on a physiological and pathological level and discuss the pros and cons of several strategies esteemed thymotrophic from a pre-clinical perspective. Finally, we will shed light on interleukin (IL)-21, a pharmacologically-promising cytokine with a significant thymotrophic nature, and elaborate on its potential clinical efficacy and safety in immune-deficient subjects.

1. Introduction

Long before its role in maintaining immune competence and tolerance surfaced, the thymus floundered through eras of different inaccurate conceptions (a mysterious/magical organ; a physiological cushion for the cardiovascular system; a risk factor for sudden infant death; and a factory producing "particles" on which "the existence of animals depended") [1]. The English (Francis Glisson/William Hewson) then acknowledged thymic susceptibility, relating the gland's persistent atrophy to aging and diseases [2]. John Beard contributed further to the lymphatic nature of the thymus, regarding it as the birth place of lymphocytes [1]. It wasn't until the late 20th century that scientists acquired a thorough insight – the one familiar to us today – into the indispensable role of the thymus in immunity [3], finally dusting off the organ a long and winding historical mystery. While the thymus is primed to generate immunologically-efficient and -tolerant T cells, it is vulnerable to different acute and chronic insults which affect both, organ structure and function [4–6]. Thereby, different strategies have been utilized in an attempt to rejuvenate atrophied thymi and re-stimulate the production of T cells, especially in old immune-deficient subjects [7]. Among several researched thymotrophic strategies whose outcomes still lack in therapeutic domains (castration, transplantation, cell-based therapies, hormones/growth factors, cytokines) [8], IL-21 stands out as a clinically-promising cytokine in the context of immune deficiency, exhibiting distinct thymotrophic properties [9,10]. In this review article, we will recapitulate a vast and evolving literature discussing the factors underlying thymic atrophy and demonstrating what thymic restoration strategies have contributed pre-clinically and what they still lack thereof. In terms of originality in the addressed field, we will be approaching several strategies with a clinical mindset. We will as well harness others' and our findings on IL-21 to provide insight on the cytokine's favorable thymotrophic profile which renders it a suitable pre-clinical competitor and a future therapeutic candidate.

2. The thymus: physiology and susceptibility to insults

The thymus is a bi-lobed gland consisting of two histologically distinct zones: a cell-studded superficial cortex and a less dense inner medulla. Enclosed within a collagenous capsule, both regions comprise epithelial cells of non-hematopoietic origins and lymphatic tissues in

Abbreviations: BM, bone marrow; BMP, bone morphogenetic protein(s); BMT, BM transplant; DN, double negative; DP, double positive; ESC, embryonic stem cell(s); Flt3L, Fms-like tyrosine kinase 3 ligand; GH, growth hormone; GVHD, graft-versus-host disease; HSC, hematopoietic stem cell(s); HSCT, HSC transplant; IGF1, insulin growth factor 1; IL-, interleukin-; ILCs, innate lymphoid cells; KGF, keratinocyte growth factor; LSK, Lin − sca-1 + c-kit +; r, recombinant; RTE, recent thymic emigrants; SCF, stem cell factor; SCT, stem cell transplant; SSA, sex steroid ablation; TBI, total body irradiation; TCR, T-cell receptor; TEC, thymic epithelial cell(s); TEPC, thymic epithelial progenitor cell(s); SP, single positive; DEX, dexamethasone

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which specific antigen presenting cells school immature thymocytes to become immunocompetent and self-tolerant – a process termed thymopoiesis [4]. Despite its vital role, the thymus confers immense susceptibility, which explains why it is the earliest and almost only organ to undergo eccentric mass loss [5]. Underlying thymic atrophy are acute and chronic factors. The former includes diet, infections, graft versus host disease (GVHD), environmental stressors (physical/emotional), pharmacological interventions (immunosuppressive and anticancer drugs), and irradiation [7,11,12]. In acute settings, stressors generally diminish cortical thymocyte counts and naïve T-cell output, whereas their removal allows a mass rebound [13]. Chronic thymic involution, on the other hand, is age-dependent. The human thymus reaches its maximum output at puberty after which it conspicuously starts to shrink and become surmounted by adipocytes throughout adulthood [14]. Therein, division of memory rather than naïve T cells in the periphery takes over [6]. Also attributable to age-induced thymic involution are sex steroid hormones, androgen and progesterone [15]. Both can lead to defective T-cell receptor (TCR) rearrangement early during thymic maturation, reduce the expression of chemokines and transcription/growth factors, and support accumulation of acute factors [6,7]. Also notable, aging tips the balance of hematopoietic stem cell (HSC) differentiation in favor of myeloid rather than lymphoid lineages and mounts the levels of memory T cells as opposed to memory B cells and naïve T cells [16]. Phenotypically, aged naïve T cells exhibit lower CD4 co-receptor expression, smaller size, reduced mitochondrial mass, downregulated activation molecules (CD25 and CD62L), and augmented inhibitory molecules (CD5 and PD-1) [6]. In general, the increased longevity of naïve T cells causes the detrimental buildup of reactive oxygen species and compromises their effector and memory cells function [17] attributing to the impaired responses to infections and reduced efficacy of vaccines in the elderly [18].

3. Strategies targeting thymopoietic regeneration

Despite not yet having a lucid notion on the interplay between acute and chronic factors and how they initiate thymic involution, numerous studies corroborate that stimulating thymopoiesis is feasible. Upcoming is a therapeutics-disciplined description of the most researched thymopoietic agents (Fig. 1) followed by an elaboration on IL-21 and its importance as a novel agent with a potential of triggering *de novo* thymopoiesis (Fig. 2).

3.1. Castration and sex steroid ablation (SSA)

Sex steroids antagonize the expression of thymic hormones by acting upon thymic epithelial cells (TEC)-expressed androgen receptors [15,19] resulting in thymic function decline [20]. Conversely, their ablation via castration or pharmacological treatments drives thymopoiesis through various mechanisms, including enhanced HSC function and CCL25/Notch ligand expression, the latter of which partakes in T lineage commitment [21]. SSA increases thymic cellularity and weight in mice and humans [22,23]. Plus, it alleviates irradiation-induced atrophy of the thymus [24] by refurbishing its microenvironment, resulting in enhanced export of recent thymic emigrants (RTE) [25]. Several FDA-approved Leutinizing Hormone-Releasing Hormone agonists which target LH and FSH generation have been reported to intensify naïve CD4 T-cell reconstitution and diversify the peripheral T cell repertoire in humans post-HSC transplant (HSCT) [26]. Contrastingly, other human studies on SSA exhibited no influence on the percentages of circulating CD4 and CD8 T cells [27,28] which insinuates further doubts about their clinical efficacy in defying immune incompetence. Other than those pilot studies performed with few patients, no major trials were run assessing the therapeutic utility of SSA in thymopoiesis.

3.2. Thymus transplantation

Patients who undergo thymus/thymic fragment transplantation are usually congenitally athymic (complete DiGeorge Syndrome) and have a life expectancy of less than 2 years [29]. Clinical efficacy requires that the donor thymus be derived from an individual aged less than 9 months and undergoing congenital heart disease surgery, during which the thymus is partially or completely excised [30]. Despite successful T-cell reconstitution seen in more than 60% of congenitally-athymic recipients [31], this procedure elicits adverse autoimmune reactions even with immunosuppressive therapy [32]. Plus, the scarcity of clinical data documenting sufficient thymic restoration in acute/chronic insults renders the procedure thymotrophically incompetent [33,34].

3.3. Cell-based therapies

Some promising cellular strategies in murine immune reconstitution include (i) OP9-DL1 system-generated precursor T cells [20], (ii) *ex vivo*-produced thymic epithelial progenitor cells (TEPC) [35], and (iii) directed reprogramming of human embryonic stem cells (ESC) and mouse embryonic fibroblasts into, respectively, thymic epithelial progenitor-like cells and TEC [36–38]. However, those strategies have their drawbacks in terms of durability [39], scalability of cortical/medullary TEC reconstitution, and potential for human extrapolation [40]. Noteworthy, a distinct subpopulation of adult TEPC comprising < 1% of total TEC was recently identified as bipotent, capable of proliferation and differentiation into both thymic epithelial lineages [41]. In a preclinical context, murine ESC derived-TEPC demonstrated enhanced thymic rejuvenation with no complications following BM transplant (BMT) in young and aged mice [42]. Other relevant approaches include *ex vivo*-engineered organs such as (i) murine embryo-derived TEC co-cultured with thymic progenitors onto dental sponges, (ii) TEC-seeded matrigels, and (iii) artificial thymic organoids [43,44]. Even though they exhibit *de novo* thymopoiesis and donor tolerance, these organs fail to preserve cell-cell contact during seeding and mimic the intricate physiological thymic compartmentalization [44,45]. Furthermore, difficulties are still encountered in finding a sustained source of TEC and establishing protocols suitable for their *in vitro* expansion to serve therapeutic purposes [44,45].

3.4. Hormones and growth factors

Given its neuroendocrine nature, the thymus is modulated by autocrine- and exocrine-derived hormones and growth factors showing thymotrophic outcomes [46].

3.4.1. KGF

Keratinocyte Growth Factor (KGF), an FDA-approved compound used as an anti-mucositis recombinant protein (Palifermin®) in cancer patients undergoing chemo/radio-therapy, is implicated in T-cell maturation by acting on TEC expressing KGF receptor [47]. In acute thymic atrophy, it prevents TEC injury and increases peripheral T-cell counts post-BMT [48,49]. KGF administration also enhances thymic regeneration in pharmacologically-induced atrophy [49]. In aged mice, it increases TEC counts and restores their organization [50]. However, when administered post-HSCT in a phase I/II study of 100 patients, it did not promote early T-cell recovery, even at high doses [51]. In another terminated trial assessing the efficacy of Palifermin® vs. placebo in reducing acute GVHD in 155 haematologically malignant patients undergoing allogeneic stem cell transplant (SCT), no significant difference in the primary outcome (severe GVHD) was recorded. Of note, this is the only registered trial with published results and shows that 64.10% of patients in the interventional arm developed severe adverse events [52].

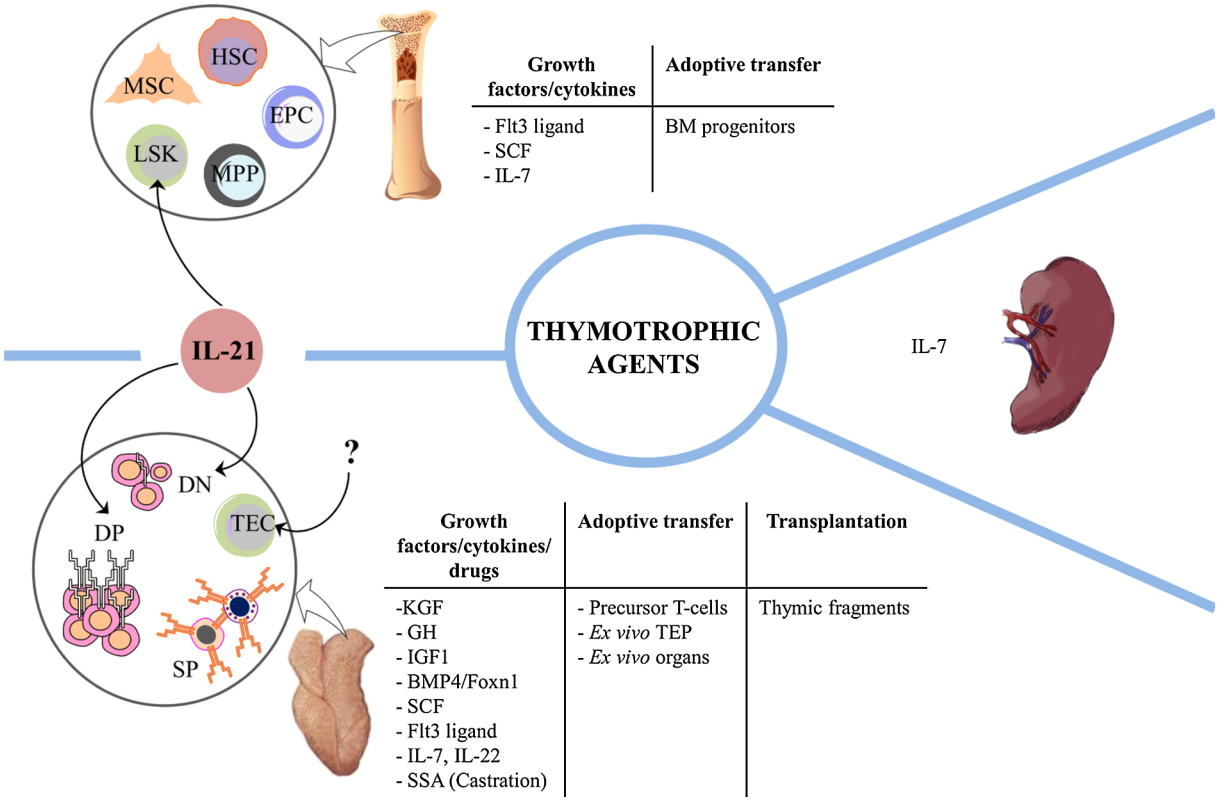

Fig. 1. Schematic diagram displaying all strategies targeting T-cell development. A list of T-cell development strategies classified according to the organ they target: BM, thymus, and spleen. For IL-21, the known effects are on LSK cells within the BM and on the thymus acting directly on DN/DP thymocytes and indirectly on TEC.

3.4.2. GH

Growth hormone (GH), whose receptor is expressed on thymocytes and TEC, modulates intrathymic hormone secretion as well as T-cell import, migration, adhesion to stromal cells, and export [8,53]. Although the administration of recombinant (r)GH after BMT in mice increases immune reconstitution and thymic cellularity, no sustained T-cell differentiation is observed [53]. With age, GH records a continuous decline, and its re-administration reconstitutes thymic mass but to levels lagging behind those of young controls [54]. Clinically, a single trial has assessed rGH safety/efficacy in enhancing immune reconstitution in a population of patients undergoing allogeneic SCT, yet it was terminated early due to low data accrual [55].

3.4.3. IGF1

Insulin Growth Factor 1 (IGF1) works in tandem with GH to maintain thymic function [56]. In fact, it confers similar thymopoietic effects to GH, regulating thymic precursor seeding and thymocyte trafficking possibly by increasing TEC counts as observed in thymectomized mice [53,57]. While IGF1 enhances thymic precursor populations and supports immune reconstitution in murine models post-BMT [58], it increases T-cell levels in a single human case [59]. Although increased T-cell counts with IGF1 may indicate *de novo* thymopoiesis, adverse effects like GVHD remain problematic [59]. In relevant settings, no clinical trials have been designed yet for testing IGF1 safety and efficacy.

3.4.4. BMP4

Bone morphogenetic proteins (BMP) belong to the superfamily of TGFβ ligands and are involved in the regulation of multiple cellular processes [60]. BMP4 partakes in TEC lineage commitment from pluripotent stem cells [37], targets thymocytes and thymic stroma, and arrests the transition of thymocytes from the double negative (DN) to the double positive (DP) stage [61]. A recent study shows that tamoxifen-induced depletion of BMP4 in murine models does not deregulate thymic function, unless followed by immediate irradiation. In the latter case, irradiation induces damage-resistant thymic endothelial cells (EC) to upregulate BMP4 and concomitantly FOXN1, a transcription factor implicated in TEC development. Following their *ex vivo* propagation, administered EC to irradiated mice promote thymic regeneration via BMP4-driven cortical TEC proliferation. However, the effects may not be exclusive to BMP4 [62]. To date, no clinical data are disclosed on BMP4 in the context of GVHD or immune restoration.

Available are other studies discussing similar preclinical effects with growth factors and hormones including, but not limited to, Leptin [63], Ghrelin [64], and Adrenocorticotropic hormone [65].

3.5. Cytokines

The rich thymic secretory profile and its functional involvement esteem cytokines a hot field for thymopoiesis research.

3.5.1. IL-7

Investigated the most, interleukin-7 (IL-7) is a stromal cell-secreted cytokine supporting thymic development and assisting T-cell homeostasis [66]. IL-7 is indispensable for expanding effector memory T cells, conserving naïve T-cell repertoire, and maintaining its diversity [67] mainly by transducing Jak-Stat/PI3K-Akt pathways, thereby activating anti-apoptotic genes, suppressing pro-apoptotic proteins, and favoring cell cycle progression [67]. In early thymocytes as well as single positive (SP) T cells, IL-7 activates the transcription factor NFATc1, promoting cell survival and development [68]. In mice transplanted with human HSC, enhanced intrathymic IL-7 signaling conditions thymopoiesis [69]. Similarly, a recombinant protein consisting of IL-7 and hepatocyte growth factor boosts thymocytes and TEC counts to levels beyond that of solely administering IL-7 [70]. In adult and old rhesus monkeys however, the administration of IL-7 sustainably expands memory but not naïve T-

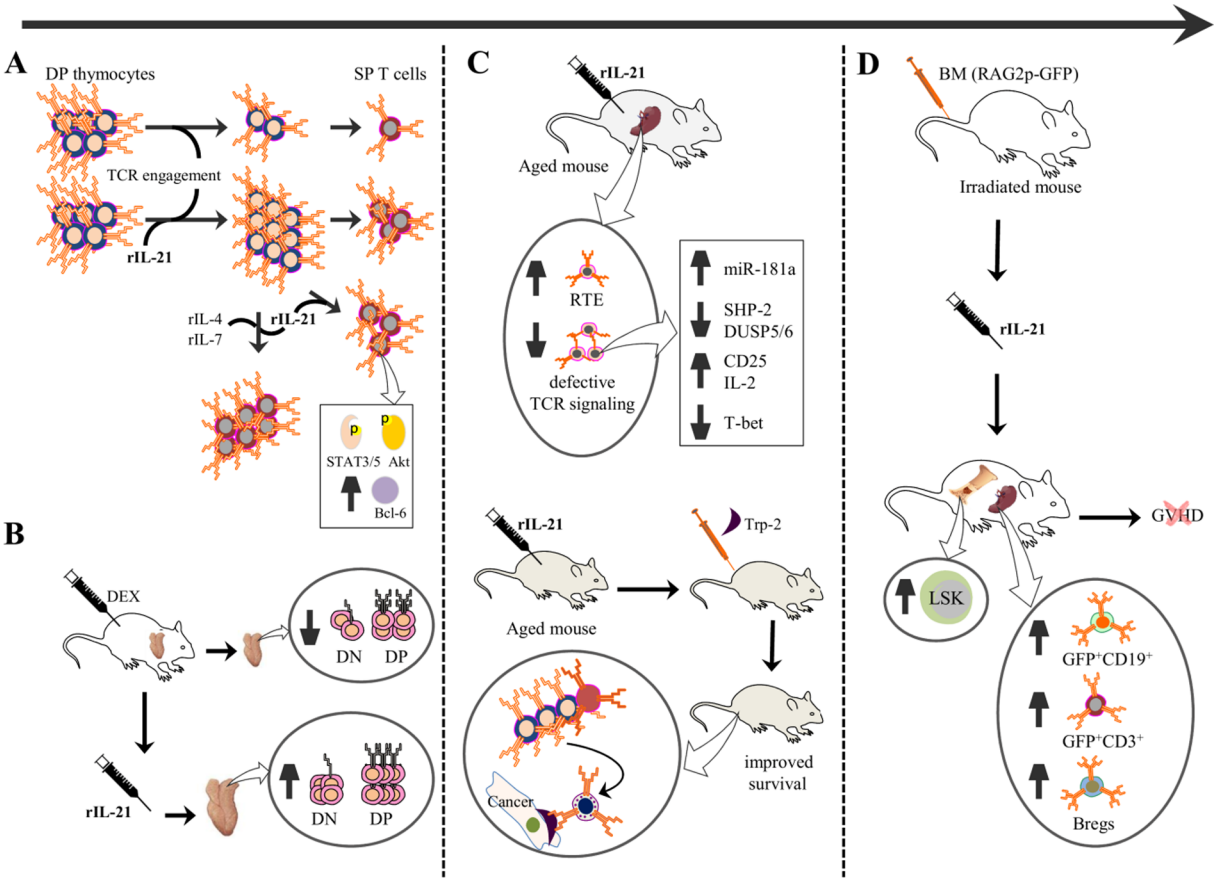

Fig. 2. Cartoon displaying the mechanism of action for IL-21 (A) In an *in vitro* differentiation system, IL-21 increases the expansion of TCR-engaged DP thymocytes. T-cell differentiation however, was dependent on IL-4, IL-7 or IL-13. (B) IL-21 administration to mice suffering from acute thymic atrophy caused by DEX administration leads to an increase in thymic size and DP/DN counts. (C) The effect of IL-21 administration on chronic thymic atrophy triggers *de novo* generation of RTE. Additionally, a decrease in defective TCR repertoire was linked to significant improvements in biochemical signaling. When used as an immune-enhancer prior to vaccination of aged mice, IL-21 improved survival confirming that enhanced *de novo* immune reconstitution of naïve T cells increases responsiveness to vaccination. (D) Reconstitution of peripheral immune populations was enhanced following IL-21 administration but was IL-7-dependent. Another critical observation in treated mice was the absence of GVHD, which was linked to increased levels of regulatory B cells. Further analyses showed improved counts of LSK suggesting a direct effect of IL-21 on the BM compartment as well.

cell counts, regardless of dose repetition [71]. Clinically, human rIL-7 predominantly expanded effector memory T-cells in human recipients of allogeneic HSCT [72]. Likewise, patients aged 23–70 and diagnosed with idiopathic immunodeficiency displayed a significant increase in their total but not naive CD4 T-cell counts upon IL-7 dosing, which questions the cytokine's thymostimulatory nature [73]. Hence, more clinical studies have yet to prove whether IL-7 can trigger immune reconstitution in a thymic-dependent manner.

3.5.2. SCF

Stem cell factor (SCF) is a stromal cell-secreted cytokine whose receptor, c-kit, is expressed by thymocyte precursors and involved in establishing lymphoid lineage during early developmental stages [74]. Following intrahepatic transplantation of human fetal liver HSC in lymphopenic mice, SCF injection elicits a four-fold increase in thymic cellularity; however, the increase is independent of the influx of BM-expanded lymphoid progenitors [75]. Even when combined with IL-7 in HSCT settings, the thymopoietic potential of SCF is further questioned especially with the observation of intact differentiation of thymocyte subpopulations in mice alongside poor thymopoiesis and high mortality risk in humans [76,77].

3.5.3. Flt3L

The cognate receptor of Fms-like tyrosine kinase 3 ligand (Flt3L) is expressed on a subset of early thymocytes as well as early BM progenitor cells [78]. *In vivo*, Flt3L treatment of mice undergoing BMT enhances thymic cellularity, thymocyte distribution, and peripheral T-cell counts [79]. This potential concurs with the expansion of $Lin^{-}sca\text{-}1^{+}c\text{-}kit^{+}$ (LSK) cells, which sparked interests for further clinical studies [79,80]. Unfortunately however, the administration of human rFlt3L (aka CDX-301) to healthy volunteers did not meet prospective results, as it led to modest HSC progenitor expansion [81]. Likewise, a pilot study on CDX-301 tolerability yet in haematologically malignant patients receiving allogeneic HSCT was terminated with no further ado (NCT02200380) [82].

3.5.4. IL-22

Primarily secreted by Th17 and subsets of innate lymphoid cells (ILCs), IL-22 serves to preserve mucosal barriers [83,84]. In acute thymic injury, it is suggested that the depletion of DP thymocytes drives IL-23 secretion by dendritic cells which trigger ILCs to produce IL-22. Resulting is STAT3/5-mediated regeneration of thymic epithelial milieu [21,85]. A study by Dudakov et al. shows that following sub-lethal total body irradiation (TBI), $IL\text{-}22^{-/-}$ mice exhibit impaired thymic restoration and diminished thymic cellularity, even with HSCT rescue [85]. In a preclinical context, the same group administered rIL-22 to mice following sub lethal-TBI and noted enhanced thymocyte and TEC counts. However, no significant thymic changes were detected with rIL-22 treatment in mice undergoing lethal TBI, unless accompanied with syngeneic HSCT [85]. More recent findings indicate that donor T-cell

subsets contribute largely to IL-22 production in recipients of allogeneic HSCT and participate in the recovery of thymocyte and TEC counts. Still, rIL-22 injection post-allogeneic HSCT failed to fully recover thymocytes on the long term and even worsened GVHD in recipient mice [86]. Clinically, an ongoing study assessing the benefit of human rIL-22 in combination with corticoids in 27 patients newly diagnosed with gastrointestinal GVHD following HSCT might provide a crude insight on the utility of IL-22 in thymic rejuvenation [87].

3.5.5. IL-21

As well as IL-10 [88], IL-21 was recently reported to display thymostimulatory properties [10,89]. Well-researched in rather oncoimmunology than thymopoiesis, IL-21 is a pleiotropic cytokine produced by T cells (CD4, CD8, NK, Tfh, and Th17) [90]. When bound to its receptor, it activates STAT1, STAT3 and STAT5 as well as PI3K/AKT pathways [10,91]. Because of its wide-spread receptor expression and vast array of targeted genes, IL-21 confers multifaceted immunomodulatory roles being either stimulatory or suppressive [92]. For instance, IL-21 was recently associated with inhibiting the antitumor capacity of γδ T cells by favoring the emergence of another subpopulation conferring an immunosuppressive phenotype [93]. In another less ventured field, the effect of IL-21 on thymocytes was first discovered by our group using an *in vitro* model (Fig. 2A).

Briefly, this model mimics T-cell positive selection and differentiation; it uses transgenic mice-derived DP thymocytes cultured on OP9 stromal cells pulsed with synthetic peptides and supplemented with γ-chain cytokines [89]. In the culture system, significant levels of IL-21 receptor were detected on TCR-engaged DP thymocytes, and IL-21 administration resulted in increased expansion of DP thymocytes and CD8 T-cell differentiation when combined with other cytokines [89]. *In vivo,* this property was then confirmed using both pharmacologically-induced acute (Fig. 2B) and chronic thymic atrophy models (Fig. 2C - upper panel). In both cases, IL-21 increased thymic size and DP/DN T-cell counts, without impairing the diversity of intrathymic and peripheral TCR repertoire, although next-generation sequencing techniques have yet to confirm the latter result [94,95]. Unattained with most aforementioned thymotrophic competitors (SSA, thymi transplant, precursor T-cells, *ex vivo* TEP/organs, KGF, GH, IGF-1, BMP4/FOXN1, IL-22), increasing DP/DN T-cell counts is essential for thymus-driven generation of naïve RTE and therefore efficient and durable immune reconstitution [96]. Furthermore, IL-21 pre-conditioning of aged mice (chronic model) improved their response to the melanoma Trp2 cancer vaccine (Fig. 2C - lower panel). More specifically, Trp2-vaccinated IL-21 treated aged mice exhibited significantly improved survival compared to PBS-treated controls, in parallel with peripheral T-cell pool rejuvenation [95]. Clinically, this potential might translate into efficient immune enhancement in old subjects undergoing vaccination. A follow-up study has also shown that IL-21 administration to mice undergoing T-cell-depleted allogeneic BMT (Fig. 2D) led to accelerated peripheral T-cell reconstitution [9]. However, unlike other thymopoietic agents which elicit a similar effect, IL-21 might confer more benefits in terms of cytokine dynamics. In other words, IL-21-treated mice displayed increased LSK counts suggesting that accelerated immune reconstitution may be in part due to augmented thymic seeding by BM-derived progenitors – a source of immune sustainability and durability [9,97]. Additionally, GVHD was absent in BMT recipients treated with IL-21, a property that renders this cytokine therapeutically favorable over most its competitors (transplantation, KGF, IGF-1, IL-22) [9]. Contradictorily, previous studies have shown that IL-21 blockade in donor T-cells improves the development of Tregs and reduces GVHD onset following BMT [98–101]. Still and all, there might be a justification to these discrepancies. Besides the type of BMT models used (complete allogeneic MHC mismatch vs. allogeneic MHC matched BM), regulatory B-cell activation/differentiation with IL-21 (Fig. 2D) [9] can explain the variability of data. Henceforth, further animal and clinical investigations are vital to understand and optimize the immune restorative effect of IL-21 in immune compromised contexts like allogeneic BMT and aging/vaccination.

4. Concluding remarks and future perspectives

The discovery of thymic susceptibility prompted the study of the thymotrophic potential of strategies like SSA/castration, thymi transplantation, adoptive cell therapies (progenitor T cells, TEPC, engineered thymi), hormones/growth factors (KGF, GH, IGF1, BMP4/FOXN1), and cytokines (IL-7, SCF, Flt3L, IL-10, IL-22/23). So far however, the loss of thymi function due to acute/chronic stressors is insufficiently addressed in clinical settings, for current agents are failing in terms of safety and durable efficacy. On the other hand, recent accumulating data endorse IL-21 as an agent with distinctive thymopoietic capacities. Not only does IL-21 administration culminate in the expansion of BM and intrathymic progenitors, but also it can restore the TEC compartment. Compared to other thymotrophic agents, this is deemed an added value, for the crosstalk between hematopoietic and non-hematopoietic compartments is essential for controlling thymic organogenesis and achieving full immune recovery [20]. These findings imply that IL-21 could be used alone or in combination with other available strategies for accelerating/stimulating thymopoiesis in HSCT settings as well as prior to vaccination in the elderly. Nonetheless, IL-21 remains a fairly new cytokine in thymopoiesis research. Thus, several questions remain unanswered. Is the BM or the thymus solely at the origin of IL-21-driven thymopoiesis, or is their intercommunication indispensable for establishing IL-21 effects? Does IL-21 promote the generation of autoreactive T cells, leading to increased susceptibility to autoimmune diseases? Would clinical trials exploiting the thymotrophic nature of IL-21 report similar safety and efficacy to that obtained previously in animal models? Further investigations are therefore warranted to unmask all underlying mechanisms and key functions of this new thymopoietin.

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6. Authorship

A. H. K. wrote the first draft of the review, and M.R. made the final edition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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