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Thymic Rejuvenation: Are We There Yet?

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Abstract

Vaccination is an appealing form of immunotherapy for frail senior patients. However, several studies have shown that in contrast to younger adults, older patients do not effectively respond to vaccines. This phenomenon is greatly attributed to immunosenescence, a hallmark of aging defined by a general decline in immunity caused by thymic involution. Historically, the study of thymic involution brought to attention several factors and components involved in thymopoiesis, as contributors to the phenomena. Depicting the underlying cause(s) of the dramatic changes in the production and properties of the naïve T-cell pool in the event of acute thymic injury or due to involution can therefore, help focus the efforts on the best strategy to reverse or overcome these hurdles. Here, we discuss some of the well-studied approaches for rejuvenating the thymus, and introduce interleukin-(IL) 21 as the most recent thymo-stimulatory agent in the field.

Keywords: thymus, thymocytes, thymic epithelial cells, thymic involution, interleukin-21

1. Introduction

With the advances in health sciences and services, the life expectancy of the general population is increasing. The U.S. Census Bureau estimates that by 2050, one quarter of the U.S. population will be over 60 years old [1]. A similar demographic trend is predicted for both developed and developing countries according to the world health organization [2]. A longer life expectancy is accompanied with the increase in chronic diseases [3], susceptibility to infections [4], prevalence of cancer [5], and certain forms of degenerative diseases [6]. All these challenges put a great burden not only on individuals, but on the whole population and health system [7]. Therefore, it is crucial to adopt new strategies aimed at keeping the aging population healthy.

Several of the prominent challenges facing the efforts to achieve healthy aging are being linked to changes in the immune system [6, 8, 9]. More specifically, the natural process of aging is concomitant with immunosenescence; a degeneration of the immune system caused by thymic atrophy or involution [10]. As a result, the production of T-cells through thymopoiesis is compromised, and the output of newly developed naïve T-cells endowed with the capacity of effectively responding to new antigenic challenges decreases dramatically [11]. For example, accumulated extrinsic and intrinsic T-cell receptor (TCR) signaling defects in aging naïve T-cells are found to impede their response to new antigenic challenges [12]. The elucidation of such changes and defects related to thymic aging can therefore help conclude that the elderly's optimal responses to vaccines that may strongly rely on the development of strategies aimed at rejuvenating their weakened thinned-out T-cell repertoire. In addition, immunosenescence compromises the patients' immunocompetency and makes the body more prone to the development of different pathological conditions such as cancer [13]. Although most cancer types can be removed by surgery followed by radiation and/or chemotherapy, these multimodality therapies are primarily effective against localized tumors as opposed to spread metastases [14]. Cancer vaccination represents therefore, an appealing alternative due to its limited toxicity compared to chemo- or radiotherapies and the ability to stimulate the host's own immune system to seek and destroy metastatic cancer cells [15]. Unfortunately, however, vaccines are less effective at older age due to immunosenescence [16]. Thus, there is an urgent need for the development or improvement of immunotherapies to stimulate and rejuvenate the immune response in the elderly population.

The knowledge of the elements involved in the deterioration of thymic function can help identify potential approaches for protecting or rejuvenating the thymus. We will be summarizing in this chapter most of the concepts related to thymus aging and discuss the most promising developments aimed at stimulating thymic function as a mean to rejuvenate elders' immunity. In addition, we will highlight the experimental models along with the outcomes of our work on IL-21 as a thymo-stimulatory agent [17–19]. Optimizing and adopting such concept will strengthen multi-disciplinary collaborations and lead to innovative IL-21-based strategies directed against two key elderly-related health problems: (i) providing superior responsiveness to protective and therapeutic vaccines, and (ii) reducing the incidence and/or relapse of cancer or severe infections in this vulnerable population.

2. Background

2.1. The thymus

Functional immunity relies on the balance between innate immunity as a first responder in the presence of an external insult and the development of a rather specific adaptive immune response [20]. Primary lymphoid organs such as the bone marrow (BM), the fetal liver, and the thymus are the major sites for lymphopoiesis, whereby the common lymphoid progenitors (CLP) proliferate and complete their differentiation into mature effector B and T lymphocytes. The uniqueness of the thymus stems from it being the sole specialized organ where the microenvironment and the cellular content are perfectly orchestrated to support the maturation

and development of T-cells [20]. This bilobed organ is located in the thoracic cavity between the heart and the sternum. Histologically, the thymus was believed to be mainly composed of a perivascular space (PVS) and a thymic epithelial space (TES) [21]. The latter is the main site for thymopoiesis and is subdivided into two anatomically and functionally distinct compartments: the cortex and the medulla [22]. These two compartments are connected by a cortico-medullary junction (CMJ), where the entry of lymphoid progenitors and the exit of mature thymocytes take place [23]. Furthermore, each of these two compartments is characterized by the presence of specific thymic epithelial cells (TECs), which guide and support thymocytes at their different differentiation and maturation stages [24]. Interestingly, the cortical (c-) and medullary (m) TECs have distinct expression profile for chemokines and cytokines, transcription factors and costimulatory molecules [25, 26]. As a result, cTECs and mTECs create a unique profile and microenvironment within the cortex and the medulla, respectively, which in turn affect the development and selection of thymocytes [25, 26]. Specifically, within the same compartment, cTECs and mTECs show heterogeneity in their markers and secretory profiles creating distinct microenvironments suitable for specific stages of thymopoiesis [26–28]. For example, cTECs express the thymoproteasome component $\beta 5t$ [29], Cathepsin L [30], in addition to thymus-specific serine protease, which facilitate the generation of major histocompatibility complex (MHC)-associated self-peptides required for positive selection [30, 31]. Moreover, it is being proposed that the crosstalk between thymocytes and different TECs is fundamental not only for supporting thymopoiesis but for maintaining the thymus homeostasis as well [24–26, 32]. Particularly, the interaction of developing thymocytes with certain TEC subsets sustains the regeneration of cTECs and mTECs [32–34].

Thymopoiesis or T-cell development starts with the settling of BM-derived early lymphocyte progenitors (ELP) in the thymus where they proliferate to give rise to early thymic progenitors (ETP) [35, 36]. Upon their interaction with cTECs, rapidly dividing ETPs undergo differentiation to the double-negative (DN) stage while initiating complex gene rearrangements to express a candidate TCR. At that stage, both CD4 and CD8 co-receptors are expressed and the resulting double-positive (DP) thymocyte undergoes positive selection; a process aimed at testing the rearrangement of a functional TCR. If successful, DP thymocytes receive a survival signal and continue their migration to the medulla [37]. Interaction of DP thymocytes with dendritic cells or mTECs is essential to sustain central tolerance as it deletes autoreactive clones leading finally to self-tolerant single-positive (SP) CD4 or CD8 naïve T-cells, which are determined by their restriction to MHCII or MHCI, respectively [38]. Newly developed recent thymic emigrants (RTEs—e.g., naïve CD4 or CD8 T cells) egress to the periphery where they complete their maturation and contribute to establishing a competent peripheral T-cell pool [39–41].

Despite its undisputed role in the development of T-cells, the thymus is commonly portrayed as a temporary organ due to its gradual decrease in size (progressive and irreversible loss of TES). This natural thymic atrophy/involution consequently results in gradual thymopoiesis decay.

2.2. Acute thymic atrophy

Through history, the thymus has been considered as a mysterious organ with no obvious function or purpose until 1961 [42, 43]. In the late 1800 and early 1900, scientists searching for the cause of unexplained sudden death of healthy babies and children labeled the thymus as

the villain [44]. The origin of the confusion started as an attempt to understand and find a cure for what is known today as sudden infant death syndrome (SIDS). For instance, several scientists observed that suddenly dying babies had a much larger thymus in comparison to normal subjects. These observations led to the establishment of a medical condition known as status thymicolymphaticus [45–47]. As a result, thousands of infants received prophylactic irradiation to reduce the size of their thymus [45–47]. Interestingly, studies conducted in the mid-1930s on cadavers from ill or malnourished patients revealed that the thymus of these subjects was indeed smaller than those of healthy individuals of the same age [47–49]. These new findings not only acquitted the thymus from any role in SIDS, but also opened the door to better understand the physiology and role of the thymus [50]. Furthermore, subjects that have undergone ‘precautions’ irradiation in their younger age were later shown to exhibit higher thyroid malignancy and breast cancer risk [51, 52].

Nonchronic thymic atrophy is the first indication for the presence of a given insult(s). There are common morphological characteristics of acute thymic atrophy such as loss of cortical thickness due to sharp decline in cortical thymocytes. Longer exposure to insults leads to thinner, irregular, and lower cortical to medulla ratio, while the CMJ becomes less apparent. Conversely, insult (s) removal is usually sufficient to reverse thymic atrophy and allow the rebound of the cortex and its contents [53–55]. Such insults include a variety of physiological factors such as pregnancy, malnutrition/starvation, stress as well as pathological factors such as infections, corticosteroids, or immunosuppressive therapy [49, 55–58]. The exact mechanism(s) and pathways involved in acute thymic atrophy upon exposure to one or more of these conditions is poorly understood and may vary [55]. For instance, the pathophysiological stress from infections upregulates serum glucocorticoids (GC) secretion by the hypothalamus-pituitary adrenal axis and pro-inflammatory cytokines levels [59, 60]. Thymocytes are highly affected by changes in GC levels, which can promote DP thymocytes apoptosis through caspase 8 and caspase 9 activation resulting consequently in lower naïve T-cell output [61, 62]. On the other hand, medical interventions such as irradiation regimens coupled with chemotherapy induce death of thymocytes, DCs and TECs [63, 64]. These interventions also affect the stromal compartment leading to slower TEC renewal [65]. This explains the delay in immune reconstitution following preparative regimen for BM transplantation (BMT), which leaves the patient immunocompromised [66]. It is therefore essential to elucidate and understand the patho-physiological factors triggering thymic atrophy in order to design strategies aimed at reversing their effect.

2.3. Chronic thymic involution

The progressive age-dependant atrophy of the thymus starts at birth and reaches its peak during puberty before continuing at a stable rate throughout life [53, 67]. The two main factors forming the basis of thymic involution are: (i) defects in the pre-thymic hematopoietic progenitor pool or their migration to the thymus, and (ii) the loss of the stromal compartment [68]. The latter factor is particularly problematic as it implies reduction of cTECs [67], loss of tissue structure, and changes in the microenvironmental niche [69]. Consequently, the body’s homeostatic compensatory system helps maintain the existing T-cell pool in the periphery through the expansion of mature T-cells [70, 71], which restricts the ability of aged subjects to benefit from new vaccines [71, 72]. Moreover, aged naïve T-cells are smaller and show defects in

antigen recognition, antigen-induced activation and in their ability to proliferate [69, 73, 74]. Such changes in the properties of naïve T-cells and the microenvironment of the thymus can also affect the negative selection process, which in turn, potentiates the emergence of autoreactive or defective T-cells [75]. Besides, the increased longevity of naïve T-cells and the accompanying accumulation of reactive oxygen species lead to reduced TCR diversity and impair the function of effector and memory T cells in the periphery [76–78] translating into weakened immune responses to infections and immunotherapies such as vaccines [79].

Several factors have been proposed to explain and understand the circumstances taking part in the involution of the thymus. One of the earliest factors to be investigated was the change in the level of sex hormones, which peaks during puberty. GCs are believed to negatively affect both TECs and thymocytes mostly by inducing their apoptosis [80–82]. In fact, surgical or chemical castration of male mice was sufficient to restore atrophied thymi back to their normal size and function [83]. However, the decline in androgen levels with age is not accompanied with a concomitant reversal of thymic involution [21], suggesting that additional players are involved in chronic thymic atrophy. Some of these factors include: (i) changes in BM-derived T cell progenitors [84], (ii) alterations in the profile of circulating cytokines and factors such as the leukemia inhibitory factor, interleukin (IL)-6, Oncostatin M, and stem cell factor [73], (iii) a blockade in TCR rearrangement, decreased proliferation and increased apoptosis of ETPs [85, 86], and (iv) a shift towards the myeloid lineage in the elderly [87].

Altogether, the aging of the immune system is an ongoing complex process and is related to numerous clinical challenges. To properly tackle these challenges, it is imperative to adequately understand and elucidate the defects in the aging immune system response. Ultimately, acquired knowledge will guide the efforts for designing counteracting therapies to rejuvenate the immune system.

3. Strategies to stimulate intrathymic T-cell development

T-lymphopoiesis remains functional at older age albeit to a limited extent [88]. Thus, enhancing thymic function in aged hosts remains a promising therapeutic goal. The thymus lacks self-renewing progenitors and relies heavily on sustained seeding with BM-derived ETPs [35]. Unfortunately, ETP numbers decline markedly with age due to increased apoptosis rates as well as reduced proliferative capacities [84, 88]. This in turn negatively impacts the delicate thymic stromal compartment, which depends heavily on cross talk interactions with thymic progenitors for its sustained survival [89–91]. As the magnitude of thymic output correlates closely with the overall number of DN and DP thymocytes [92], pre-conditioning of aged subjects to enhance the development/recovery of these thymic subsets is critical to actively stimulate *de novo* intrathymic naïve T-cell development.

In an attempt to reverse/block this vicious thymic involution cycle, a variety of strategies including cytokines, growth factors, hormonal therapies, in addition to adoptive transfer of precursor T cells or castration have been tested and reviewed in the literature [93–96]. However, few of these interventions stood out as the most propitious including: (i) sex-steroid ablation (SSA), (ii) keratinocyte growth factor (KGF), (iii) ghrelin (GRL), (iv) IL-7 as well as, and (v) IL-21.

3.1. SSA: to reverse aging-induced changes in the thymus

Steroid hormones elicit their effect in the body by interacting with their specific receptors, where they translocate to the nucleus and directly affect gene transcription [97, 98]. In the thymus, both thymocytes and TECs express receptors for steroid hormones and are affected by changes in their levels [99–101]. For example, testosterone induces DP thymocyte apoptosis through tumor necrosis factor- α upregulation, whereas estrogen binding to receptors expressed by DN thymocytes inhibits their proliferation [100, 102]. Due to the concurrent decline in the size of thymus around puberty, sex steroids are being widely studied as a main factor in thymus involution. For instance, studies designed to evaluate the effect of androgens on T-cell output showed that androgens do indeed have a strong effect on the output of T-cells [94, 101]. Specifically, the reported decline in thymic output is mediated mainly by the sensitivity of TECs to androgens [101–103]. Therefore, adopting a plan to promote thymic activity by antagonizing or preventing the effect of sex hormones on the thymus is perceived as both rational and reasonable [96]. SSA can indeed be achieved either surgically or chemically. Both approaches show positive impact on thymic function, cellularity, as well as architecture, and can significantly enhance thymopoiesis [96, 104]. For instance, castration conducted in different animal models resulted in an increase in thymic output, T-cell responses and helped sustain the size and function of the thymus [105–108]. More specifically, surgical castration in old mice led to rapid reversal of thymic atrophy and restoration of SP and DP thymocyte to comparable levels in young mice [107]. In addition, this approach increased T-cell responsiveness and facilitated the immune recovery of aged mice after chemotherapy and/or BMT [96, 109]. On the other hand, chemical SSA offers a transient and reversible effect by either targeting the upstream signaling events or by directly blocking steroid receptors [96, 110]. Therapeutic agents initially developed for prostate and uterine cancer such as the luteinizing hormone releasing hormone agonist or androgen receptor blocker increased both lymphoid and myeloid progenitors and accelerated thymic recovery after allogeneic BMT [102, 104]. Clinical studies on SSA have further shown increased repertoire diversity in the CD4 and CD8 T-cell populations [111]. Collectively, these preclinical data validated the idea that using SSA to enhance thymic function could lead to beneficial outcomes in both aged and immunocompromised patients [112].

Overall, SSA has a profound positive effect on the thymus. However, the long-term systemic effects of both surgical and chemical SSA are neither yet clear nor are the potential side effects. Therefore, more in depth investigations are required to assess the safety of SSA-based therapy. Moreover, the failure to restore thymic function by the natural decline in sex hormones levels in senile patients provides an evident that thymic involution is a complex process [21, 113]. Therefore, a more comprehensive approach involving different factors and mechanisms should be further investigated.

3.2. KGF: to guard the thymic epithelium

Also known as fibroblast growth factor 7, KGF is a member of the fibroblast growth factor family. It is mainly produced by mesenchymal cells [114, 115] and exerts its activity through binding to its receptor fibroblast growth factor R2-IIIb (FgfR2-IIIb or KGFR) [116]. KGF has a notable effect on triggering the proliferation of epithelial cells [116]. Therefore, Palifermin[®], a

truncated form of KGF is clinically prescribed for patients undergoing stem cell replacement after high-dose chemotherapy and radiation [117]. Specially, Palifermin[®] is administered to reduce the incidence and duration of mucositis; an inflammation and ulceration of the mucous membranes lining the digestive tract [117].

In the thymus, both TECs and thymocytes produce KGF. However, only TECs express the KGFR, which explains the protective effect of KGF on thymic epithelium [116, 118]. Furthermore, studies conducted on KGFR-deficient mice support a major role for KGF in the thymus. Particularly, these deficient mice, which die at birth due to the absence of functional lungs, present with severely hypoplastic thymus [119]. In the same context, KGFR deficiency is found to be associated with significant defects in thymopoiesis accompanied by a decrease in thymic cellularity, in addition to signs of TEC proliferation blockade [119, 120]. Interestingly, the protective effect of KGF was evaluated in mouse models for allogeneic BMT where KGF pre-treatment was found to enhance the immune development post-BMT by improving the function of thymic microenvironment [118]. In the same study, Min et al. reported that KGF pre-treatment did improve thymopoiesis and positively affect the functional T-cells pool in the periphery [118].

As KGFR is expressed by several organs targeted by alloreactive T-cells, it was compelling to evaluate the effect of KGF in the setting of acute graft-versus-host disease (GVHD). In fact, current data have shown that KGF administration post-allogeneic BMT can: (i) facilitate alloengraftment [121], (ii) alleviate GVHD [122–124], (iii) protect epithelial cell in the gut mucosa while enhancing its repair [125], (iv) reduce the release of inflammatory cytokine [126], and (v) diminish allogeneic T-cell responses [121, 127]. A subsequent study by Berent-Maoz et al. further demonstrated that KGF not only protects TECs, but could also stimulate thymopoiesis indirectly by triggering them to secrete soluble factors [128]. Conversely, data from two separate phase I/II clinical trials conducted on patients undergoing allogeneic BMT revealed ameliorated mucositis with no significant improvements on the incidence and severity of acute GVHD, T-lymphopoiesis, infections, overall survival, or cancer relapse rates [129, 130]. As such, KGF can improve mucotoxicity following allogeneic BMT without exhibiting beneficial outcome on immune recovery in BMT patients.

3.3. GRL: increasing thymopoiesis appetite

The polypeptide hormone GRL, also named the hunger hormone, is normally released by specialized cells within the stomach into the circulation where it induces hunger and the release of growth hormone by stimulating the hypothalamus and the pituitary gland, respectively [131]. Moreover, GRL is known to play an important role in maintaining energy homeostasis [132, 133]. Interestingly, the GRL receptor (GRLR) is expressed by the pituitary gland, the central nervous system as well as on various immune cells including resting and activated T-cells [134]. Moreover, GRL deficiency in mice is associated with reduced thymopoiesis and increased thymic adiposity [135, 136]. Therefore, a potential role for GRL has been proposed in reversing age-related thymic involution [95]. In fact, scientists believe that GRL represents the next generation of hormonal-based rejuvenation therapy due to its astonishing effect on the aging thymus. Specifically, GRL administration to aged mice (14–22 months) resulted in improved TCR diversity, increased thymic cellularity (including cTECs, mTECs, and ETPs) and RTE output [134, 137]. In addition, Dixit et al. demonstrated that GRL administration

inhibits adipogenesis and production of pro-inflammatory cytokines in the thymus; two important characteristics of thymic involution [134, 135, 138]. Despite these promising pleiotropic thymopoietic-stimulating effects, the progressive loss of GRLR expression with aging overshadows the potential use of GRL as a thymo-stimulatory therapy [134].

3.4. IL-7: a toolkit for thymus rejuvenation

Discovered in 1988 as a stimulator for murine B-cell progenitors, IL-7 is a member of the type-I-cytokine-family and signals through its heterodimer IL-7 receptor (IL-7R) [139]. Unlike other cytokines, IL-7 is mainly produced by nonhematopoietic cells [140]. Soon after its discovery, the profound and nonredundant effect of IL-7 on T-lymphopoiesis was underlined as it could: (i) enhance the expansion of naïve peripheral CD4 and CD8 SP T-cells [141], (ii) ameliorate the antiviral/antitumor activity of cytotoxic T-cells [142, 143], and (iii) support the survival/proliferation of CD8 memory T-cells [143–145]. These unique properties made IL-7 a central research topic in the context of viral infections, cancer therapies, and in T-cell reconstitution following BMT [140, 145]. However, the outstanding role of IL-7 as the cornerstone of T-lymphopoiesis in preclinical studies was challenged by contradicting observations made in higher species. For example, data from an autologous hematopoietic stem cell transplantation study in nonhuman primates [146] and from two IL-7 phase I clinical trials conducted on cancer patients failed to provide evidence for significant *de novo* thymopoiesis [147, 148]. This discrepancy with the results obtained from rodent models indicates the need to further elucidate the role of IL-7 in higher species. Nonetheless, IL-7 may still be clinically relevant as a potent adjuvant to stimulate T-cell effector functions in various illnesses.

3.5. IL21: a new thymostimulatory agent

IL-21 is the most recently identified member of the type-I-cytokine-family [149]. Produced mainly by activated CD4 T-cells [150], IL-21 was found to: (i) promote CD4 T-cell differentiation down the Th17 pathway [151], (ii) co-stimulate activated NK and CD8 lymphocytes [152], (iii) desensitize responding cells to the inhibitory effects of regulatory T-cells [150], and (iv) act as a switch for IgG production in B-cells [153]. In addition, similar to other type-I-cytokine-family members, IL-21 signals through its heterodimer IL-21 receptor (IL21-R), which is expressed by different hematopoietic cells such as, natural killers, B and T lymphocytes [154]. Moreover, we have recently showed that *in vitro* peptide-mediated TCR-engagement triggers potent cell surface expression of the IL-21R on DP thymocytes [18]. Although not required for hematopoiesis, BM progenitors expanded in response to IL-21 overexpression *in vivo* [155]. Likewise, IL-21 did not seem to be essential for thymopoiesis due to normal T-cell development in IL-21R^{-/-} mice [149]. Moreover, IL-21 supplementation to positively selected DP thymocytes did not trigger their differentiation to CD8 SP T-cells as did IL-7 [156]. Instead, it led to DP thymocyte expansion and a 3–4-fold increase in the absolute number of *in vitro* differentiated CD8 T-cells when combined with other differentiation-inducing cytokines such as IL-4, IL-7, or IL-13 [157, 158]. These findings prompt further investigations using three pre-clinical settings with impaired thymopoiesis: (i) ensuing pharmacologically-induced thymic atrophy, (ii) in age-related thymic involution, and (iii) for T-cell reconstitution following allogeneic BMT.

So far, no treatments are available to protect against acute thymic atrophy or to accelerate thymic recovery under such circumstances, thereby leaving the immune system compromised. Of note, sepsis caused by bacterial infections can trigger GCs (corticosterone in rodents and cortisol in humans) secretion by the hypothalamus-pituitary adrenal axis. As a result, DP thymocytes undergo apoptosis due to their inability to express the anti-apoptotic molecule Bcl-2 [82]. Such acute thymosuppressive state can be easily replicated in mice via injection of the synthetic corticosteroid dexamethasone (DEX) [159]; hence, the utility of this model in evaluating the capacity of IL-21 in accelerating thymic recovery. One day following intraperitoneal (IP) injection of wild-type C57Bl/6 mice (4–6 months old) with PBS or DEX, IL-21R was found to be expressed on total or fractionated DN as well as CD4/CD8 SP thymocytes in both animal groups [18]. In contrast, DP thymocytes expressed the IL-21R exclusively following DEX injection [18]. To further ascertain these observations, a functional *in vitro* proliferation assay was conducted using sorted DN and DP subsets. Cell counts revealed that DN thymocytes derived from PBS or DEX-treated mice proliferated similarly in a dose-dependent manner in response to IL-21 [18]. In contrast, only positively selected (CD69⁺) or DEX-derived DP thymocytes expanded in response to IL-21 [18]. To directly assess its effect on thymopoiesis *in vivo*, DEX-treated mice were IP-injected with IL-21 versus equivalent volume of PBS. Thymi derived from the IL-21 group displayed accelerated size and cellularity recovery compared to control DEX/PBS-injected animals. Analysis of the percentages obtained by flow-cytometry revealed that IL-21 treatments enhance the recovery of DP thymocytes. The response to IL-21 administration also culminated in a noticeable remodeling of the thymic architecture [18]. Furthermore, extrapolation of absolute numbers using flow-cytometry percentages revealed a significant increase in the DN and DP populations within the IL-21 group with no observed effects on the TEC compartment. This is not surprising as TECs do not express the IL-21R. To confirm that IL-21-mediated effects are due to proliferation as opposed to survival of thymic progenitors, DN and DP thymocytes were analyzed for their expression of the anti-apoptotic molecules Bcl-2 (expressed only in DN thymocytes) and Bcl-XL (expressed normally in both DN and DP thymocytes) [18]. Interestingly, none of these anti-apoptotic proteins were up-regulated in both populations supporting the notion that IL-21 administration leads to thymocyte expansion as opposed to their enhanced survival. Pertinent to this project, IL-21 administration did not skew the TCR repertoire diversity as T-cells derived from PBS, DEX/PBS-, or DEX/IL-21-treated mice displayed comparable TCRV β distribution within intrathymic and peripheral CD4/CD8 SP T-cell populations. This point is particularly important as it clearly demonstrates that IL-21 administration under acute thymic atrophy does not lead to non-specific mono- or oligoclonal T-cell proliferation. Finally, IL-21 signaling is characterized by the preferential activation of the JAK-STAT pathways [160]. Biochemical responsiveness of sorted thymic subsets in response to IL-21 stimulation *in vitro* clearly demonstrated phosphorylation of STAT1, STAT3, and STAT5 in all thymic subsets except for DP thymocytes; unless they were derived from DEX-treated mice. This was expected as nonpositively selected (CD69⁻) or nonDEX-treated DP thymocytes do not express the IL-21R [18].

In contrast to acute thymic atrophy, chronic age-related involution is characterized by a gradual expansion of the PVS and reduction of the stromal compartment capable of supporting thymopoiesis [74]. To further explore the thymopoietic potential of IL-21 in such model,

assessment of IL-21R expression with aging was conducted to discard the possibility that IL-21R expression declines progressively akin to the GRLR situation [134]. Analysis performed on total and fractionated thymocytes (DN, DP, and SPs) revealed that IL-21R expression profiles are comparable between young and aged mice (unpublished data). Next, wild type-aged C57Bl/6 mice (14–18 months old) received three IP injections of IL-21 prior to their analysis on the following day. This treatment led to increased thymic size, weight, and cellularity. Analysis of thymic subsets showed a drastic increase in the proportion of DN thymocytes as it reached 15% in contrast to 3% in PBS control mice. Likewise, a notable increase in the frequency of CD4 and CD8 SP T cells was also observed in the IL-21 group. This outcome was further reflected in the overall absolute number of DN, DP, and SP thymocytes. As one of the predominant deficiencies occurring with chronic thymic atrophy is decreased migration, proliferation, and survival of ETPs, we assessed the effect of IL-21 administration on the scarcity of this central progenitor population in the thymus. Flow-cytometry analysis of ETPs showed a significant increase in their frequencies within the IL-21 group suggesting preferential expansion of ETPs in response to IL-21 administration. As the aim of using IL-21 focuses on rejuvenating T-cell immunity of aged mice, T-cell output was analyzed weekly in PBS- vs. IL-21-treated aged mice over a total period of 3 weeks. Interestingly, aged mice undergoing IL-21 treatment showed distinctively improved T-cell responses [18]. In addition, while IL-21 administration had no impact on the absolute number of all peripheral lymphocytes, the frequency of peripheral GFP⁺ T-cells increased substantially in comparison with control mice [18]. This can also explain the improved anti-tumoral response observed in IL-21-pre-conditioned aged mice prior to cancer vaccination [18]. The sum of these results serves as the basis to investigate the use of IL-21 as an elderly pre-conditioning therapy to rejuvenate immunity, and thus, improve T-cell responsiveness to vaccination.

The above findings provided the impetus to investigate the use of IL-21 in T-cell reconstitution post-BMT. Although this medical procedure is adopted as a life-saving procedure for specific malignant and non-malignant conditions [161], it remains unfortunately associated with dangerous life-threatening complications. The high morbidity and mortality associated with BMT persist as a major clinical problem associated with increased risks of: (i) relapse or development of secondary malignancies [162, 163], (ii) infection [162, 164, 165], and (iii) reduced responsiveness to vaccination [166]. The primary factors associated with these complications are the significant delay and improper reconstitution of T-cells post-BMT [165, 167] mostly due to the significant damage inflicted to TECs [168–171]. Therefore, the ability of IL-21 to improve *de novo* T-cell reconstitution post-BMT was investigated. For this purpose, T-cell-depleted RAG2p-GFP-derived BM cells (H2-K^b) were transplanted into irradiated LP/J (H2-K^{b/c}) recipient animals followed by IL-21 administration. In this experiment, GFP expression driven by the Rag2 promoter allows direct assessment of *de novo* peripheral T-cell reconstitution [172]. Analysis of transplanted mice revealed that IL-21: (i) accelerates lymphocyte reconstitution including T-cells (also observed in NOD scid gamma (NSG) mice receiving human IL-21 following cord-blood transplantation), (ii) slightly improved TECs recovery, (iii) regenerates a naïve peripheral T-cell pool with a diverse TCR repertoire, (iv) enhances regulatory B-cell development, and (v) protects from GHVD while retaining the graft-versus-tumor effect. Of note, IL-21 was originally believed to specifically affect the thymus [154]. In line with a

previous study, however, BM analyses conducted on transplanted mice revealed increased counts of Lin⁻Sca1⁺cKit⁺ (LSK) cells in IL-21-treated mice [17, 155]. This observation is particularly interesting as HSC/HSC progenitors capable of generating lineages of the hematopoietic system are enriched within the LSKs population [173].

4. Conclusion

So far, the use of IL-21 in the clinic remains limited to cancer immunotherapy (IL-21-based stimulation) or in the setting of autoimmune diseases (IL-21 inhibition). The latter clinical objective is particularly important as it raises major concerns related to the use of IL-21 in patients prone to develop autoimmune ailments. Therefore, additional studies are warranted to specifically address the clinical use of IL-21 in indications such as immunotherapies. Alternatively, all pre-clinical observations related to IL-21 strongly suggest that this cytokine could be exploited either as a monotherapy or in combination with other standards of care. This would ensure, at least in the context of thymopoiesis, improved *de novo* T-cell development in aged subjects as a mean to reverse thymic involution or post-BMT to accelerate the regeneration of naive T cells.

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