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Review

Molecular control over thymic involution: From cytokines and microRNA to aging and adipose tissue

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The thymus is the primary organ for T-cell differentiation and maturation. Unlike other major organs, the thymus is highly dynamic, capable of undergoing multiple rounds of almost complete atrophy followed by rapid restoration. The process of thymic atrophy, or involution, results in decreased thymopoiesis and emigration of naïve T cells to the periphery. Multiple processes can trigger transient thymic involution, including bacterial and viral infection(s), aging, pregnancy and stress. Intense investigations into the mechanisms that underlie thymic involution have revealed diverse cellular and molecular mediators, with elaborate control mechanisms. This review outlines the disparate pathways through which involution can be mediated, from the transient infection-mediated pathway, tightly controlled by microRNA, to the chronic changes that occur through aging.

Keywords: Aging • Animal Models • Epithelial Cells • Lymphoid Organs • T Cells

Introduction

The thymus is the primary organ for thymocyte differentiation and maturation into functional T cells. During the process of thymic involution, the size and function of the thymus is dramatically reduced, with changes in the order of a 95% reduction frequently observed. This involution results in decreased thymopoiesis and emigration of naïve T cells to the periphery. There are multiple causes that have been linked to thymic involution, which can loosely be grouped into categories of external stimuli and normal physiology. The external stimuli that cause thymic involution include infection and various physiological and psychological stresses, while the normal physiological processes that cause thymic involution are those of pregnancy and aging.

The evolutionary reason for thymic involution is still a matter of considerable debate [1, 2]. Several valid hypotheses have been generated which postulate either metabolic or immunological rationales for the process. The metabolic hypotheses generally concentrate on the energy expense required to maintain thymopoiesis, and thus the advantage to be gained by cessation of thymic function during periods of infection or stress, during the metabolically expensive process of pregnancy or during the suboptimal resource environment of aging [3]. Alternatively, the immunological hypotheses postulate risks incurred by continued thymocyte differentiation under these conditions, such as the possibility of generating regulatory T cells against infectious antigens, producing effector T cells against fetal antigens, or introducing stochastic variation into an already optimized repertoire in the context of aging [1, 2]. As thymic involution is an evolutionarily ancient process [3], for the purposes of this review we will assume that either or both of these rationales are correct, and thus concentrate instead on the molecular mechanisms by which involution is driven.

In order to maintain a structural environment able to support the normal differentiation of thymocytes, complex interactions are required between thymocytes and the structural components of the thymus, namely thymic epithelial cells (TECs), endothelial cells, and mesenchymal fibroblasts. In principle, each of these cell types could act as a mediator of thymic involution, as a programed cessation of function in each would drive thymic involution, although known mechanisms are largely mediated by either thymocytes or TECs. While these two cellular populations are both able to mediate thymic involution, the mechanism by which they do so is quite distinct. Thymocyte-mediated thymic involution is a largely apoptotic process, whereby the double positive (DP) thymocytes that make up the bulk of thymic cellularity rapidly die,

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creating a shrunken thymus and a block in thymocyte differentiation [4]. By contrast, TEC-mediated thymic involution appears to be a more ordered process, and results in a thymus of reduced cellularity capable of maintaining normal thymocyte differentiation [5]. Current research suggests that thymic involution due to infection and pregnancy is largely TEC-dependent while involution due to stress (including stress due to infection) is thymocyte-mediated and involution with age is multifactorial. The molecular mediators of each will be discussed in turn.

Thymic involution in response to infection

The thymus is highly responsive to acute involution following infection, as multiple infectious stimuli have been demonstrated to trigger involution in both humans and mice [6–10]. This is thought to be advantageous, as the cessation of T cell production during infection reduces the likelihood of dominant tolerance being induced toward pathogen-associated antigens, protecting the infectious agent from clearance [11]. Two distinct mechanisms can be responsible for thymus involution during infection: (i) an infection-sensing pathway, where TECs drive programed thymic involution and (ii) an inflammation-mediated pathway, where the byproducts of infection cause direct apoptosis of thymocytes. The first pathway is described here, while the second pathway is shared by multiple other stressors and is outlined in "Stress-mediated thymic involution."

The ability of the thymus to respond to infection signals prior to the buildup of inflammatory mediators is dependent on the recognition of pathogen-associated molecular patterns. While multiple pathogen-associated molecular patterns may be able to directly trigger thymic involution, most research has been performed using poly(I:C), which is structurally similar to the dsRNA of viruses. Mice injected with poly(I:C) undergo rapid thymic involution within several days, in a process which is mediated through the virus-sensing melanoma differentiation-associated gene 5 (MDA-5) receptor. Both the expression of MDA-5 and the interferon α receptor (IFNAR) are required for thymic involution, as loss of either gene prevents thymic involution in response to poly(I:C), demonstrating that the involution pathway works via poly(I:C) binding MDA-5 and in turn triggering IFN-α expression [12]. Of several putative sources of IFN-α in the thymus, a key candidate is plasmacytoid dendritic cells, which have the capacity to produce very high titres of IFN-α in response to CpG [13]. In vitro human experiments have shown that IFN-α produced by plasmacytoid dendritic cells inhibits T-cell development [13], however this is unlikely to be a direct effect as in vivo murine experiments show that the involution-inducing effect of IFNAR is independent of IFNAR expression by DP thymocytes or thymic dendritic cells [12], suggesting that the TEC component of the thymus responds to IFN-α and transmits the involution signal. The primary effect on developing T cells is a block in the DN1–DN2 transition, resulting in decreased DN4 and DP T cells [14].

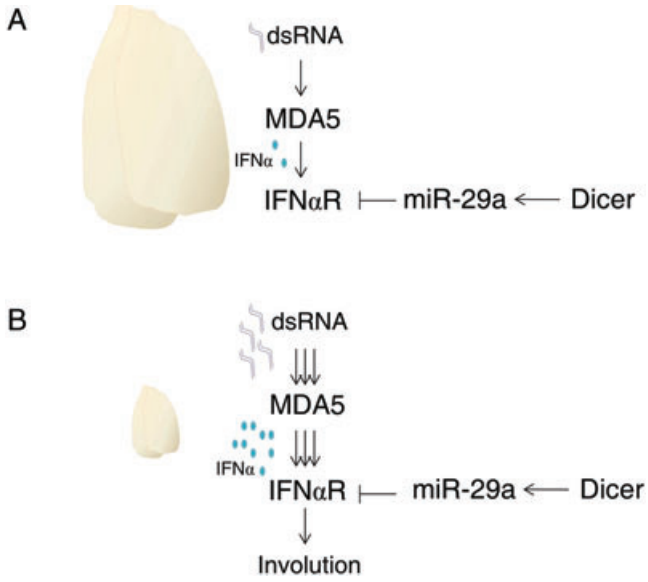

Figure 1. Molecular regulation of infection-associated thymic involution. (A) Exposure to commensal microflora results in the basal activation of **MDA**-5 and low levels of **IFN**-α production. However, due to the activity of **D**icer-dependent miR-29a, **IFNAR** levels on **TEC**s are kept low enough to prevent triggering the involution pathway. (B) By contrast, during an infection, levels of pathogen-associated molecular patterns are high enough to induce the production of sufficient **IFN**-α to activate **IFNAR** on **TEC**s. The **TEC**s, in turn, drive an active involution process, transiently reducing thymic cellularity.

Recent research demonstrates that this poly(I:C)-driven thymic involution is under tight control by microRNA (Fig. 1). Loss of either the complete microRNA network (through deletion of Dicer) or the miR-29a cluster in TECs results in elevated sensitivity of the thymic epithelium to poly(I:C) and premature chronic thymic involution [15]. This effect is mediated through IFNAR, which is a direct target of miR-29a and is upregulated on the thymic epithelium of Dicer-deficient or miR-29a-deficient mice [15]. The evolution of a tight control of IFNAR expression in TECs is likely to be a response to the altered function of IFN-α signaling in TECs in comparison with non-TECs; while non-TECs respond to IFN-α by maintaining cellular integrity and increasing innate resistance to infection [16], TECs are programed to reduce functionality and suspend thymopoiesis. miR-29a expression is therefore required to dampen down the sensitivity of TECs, by suppressing IFNAR expression, in order to restrict thymic involution to the higher magnitude increases in IFN-α production that indicate major infection, rather than the low magnitude increases in production that are probably associated with hosting commensal microflora.

Age-related thymic involution

Thymic size and function is at its maximum during fetal and perinatal development, and then displays a chronic progressive decline with age. The major feature of thymic involution is a systematic loss of thymocytes and TECs; however, architectural

changes are also observed, such as a breakdown of the cortical-medullary junction [5] and a shift in the TEC ratio toward the cortical subset [17]. Age-related involution of the murine thymus eventually reduces cellularity to less than 5% of its adult peak [5], while age-related thymic decline in humans has been estimated to be in the order of ~1% per year [18]. Despite the decline in thymus cellularity, its function is maintained at a level that is proportional to the reduced size, with no blockade in thymocyte differentiation being observed [17]. Intriguingly, overexpression of the master transcription factor of TEC fate, Foxn1, is able to diminish the effects of age-related thymic involution [19].

Sex hormones and age-related thymic involution

Age-related thymic involution is often described as being sex hormone dependent. Both male and female sex hormones, androgens and estrogens respectively, have been directly associated with thymic involution [5]. Sex hormone ablation by chemical or surgical castration reverses age-related thymic involution and restores thymic function with a decrease in the rate of thymocyte apoptosis and increased proliferation of both T cells and TECs [5, 20]. While the reproductive organs express multiple important immunological factors, such as IGF-1 (insulin-like growth factor 1), PDGF, Nerve Growth Factor (NGF), and TGF-β [21], the effects of chemical/surgical castration are thought to be mediated primarily by androgens or estrogens, as in the reciprocal experiment administration of these sex hormones to uncastrated mice results in decreased thymopoiesis, with an increased rate of apoptosis of immature thymocytes [20], although further work is necessary to prove this. The effect of sex hormones on thymic involution is mediated by TECs, as TEC expression of androgen/estrogen receptors is required for thymic involution and normal thymic development [22].

Despite this evidence that sex hormones are potent mediators of thymic involution, a number of lines of evidence suggest that they are not the primary mediators of age-related involution. First, while sex hormone ablation reverses age-related thymic involution it also boosts the function of a normal, nonaged, thymus, suggesting a generic suppressive effect of sex hormones rather than an age-dependent effect [15]. Second, progressive age-related thymic involution is not mirrored by progressive increases in sex hormone levels, which can actually decline in age [23]. Third, "age-related" thymic involution appears to be initiated before puberty [3]. Fourth, the boost to thymic cellularity that occurs due to sex hormone ablation is transient and, if performed in young mice, does not prevent age-related decline [24]. Together these results suggest that a sex hormone-independent mechanism mediates age-related thymic involution, which is nevertheless responsive to the thymic growth that occurs following ablation of sex hormones.

Adipocyte expansion and age-related thymic involution

An alternative model to explain age-related thymic involution is based on the effect of a buildup of adipocyte tissue. Unlike sex hormones, adipocyte tissue generally increases steadily with age, which is accompanied by an expansion of lipid-bearing cells within the medulla of the thymus [25]. The ability of adipocytes to drive thymic involution is suggested by the premature thymic involution exhibited by obese mice with mutations in leptin (ob/ob mice) or the leptin receptor (db/db mice) [26]. Additionally, administration of leptin to ob/ob mice results in weight loss and increased thymopoiesis [26]. Leptin receptors are expressed on TECs [27] and can modulate cytokine expression [28]; however, it is likely that the effects of leptin loss on thymic involution are due to obesity rather than direct leptin signaling, as the addition of adipocytes in vitro or adipocyte explants in vivo compromises T-cell differentiation [25, 29]. Conversely, inhibiting thymic adipogeneis by calorie restriction has been shown to reduce age-related thymic involution and increase thymic function [25, 30]. The critical fat deposits for thymic involution appear to be those located nearest to the thymus, as the highest effect of adipocyte explants is observed when transplantation occurs close to the thymus [29]. This suggests that adipocytes are actively contributing to thymic involution and are not just innocent bystanders.

The mechanism of adipocyte-mediated thymic involution is likely to be multifactorial, driven by the removal of positive factors, such as SCF, FGF7, FGF10, and VEGF, the production of negative factors, such as leukemia inhibitory factor (LIF), oncostatin M (OSM) and IL-6, and anatomical changes. The removal of positive factors may occur because thymic adipocytes develop at the expense of thymic fibroblasts, as the latter become lipid-laden with age and transition into adipocytes [25]. Thymic fibroblasts express critical thymic growth factors that are essential for thymocyte (SCF), TEC (FGF7 and FGF10), and thymic endothelial cell (VEGF) maintenance [31]. The importance of fibroblasts in maintaining thymic cellularity is demonstrated by thymic grafts in which the mesenchyme-derived populations are removed; these fibroblast-depleted grafts support normal T-cell development but the thymi are reduced in size [32]. Likewise, FGFR2IIb-deficient mice and FGFR2IIIb-dominant negative mice, that is, mice that lack FGF7 and FGF10 signaling, have a reduced thymic size [33, 34]. Conversion of fibroblasts to adipocytes may also be responsible for the deficiency in IGF and growth hormone, which reduces thymic function [35, 36]. Thus, the transition of thymic fibroblasts to adipocytes may result in the reduction of critical support factors, causing thymic involution (Fig. 2).

The increase in adipose tissue with age also results in the increased production of negative factors for thymic maintenance (Fig. 2). Adipocytes have been show shown to produce thymus-suppressing factors, including LIF, OSM, and IL-6, sex hormones, and steroids. These factors have been shown to increase in the

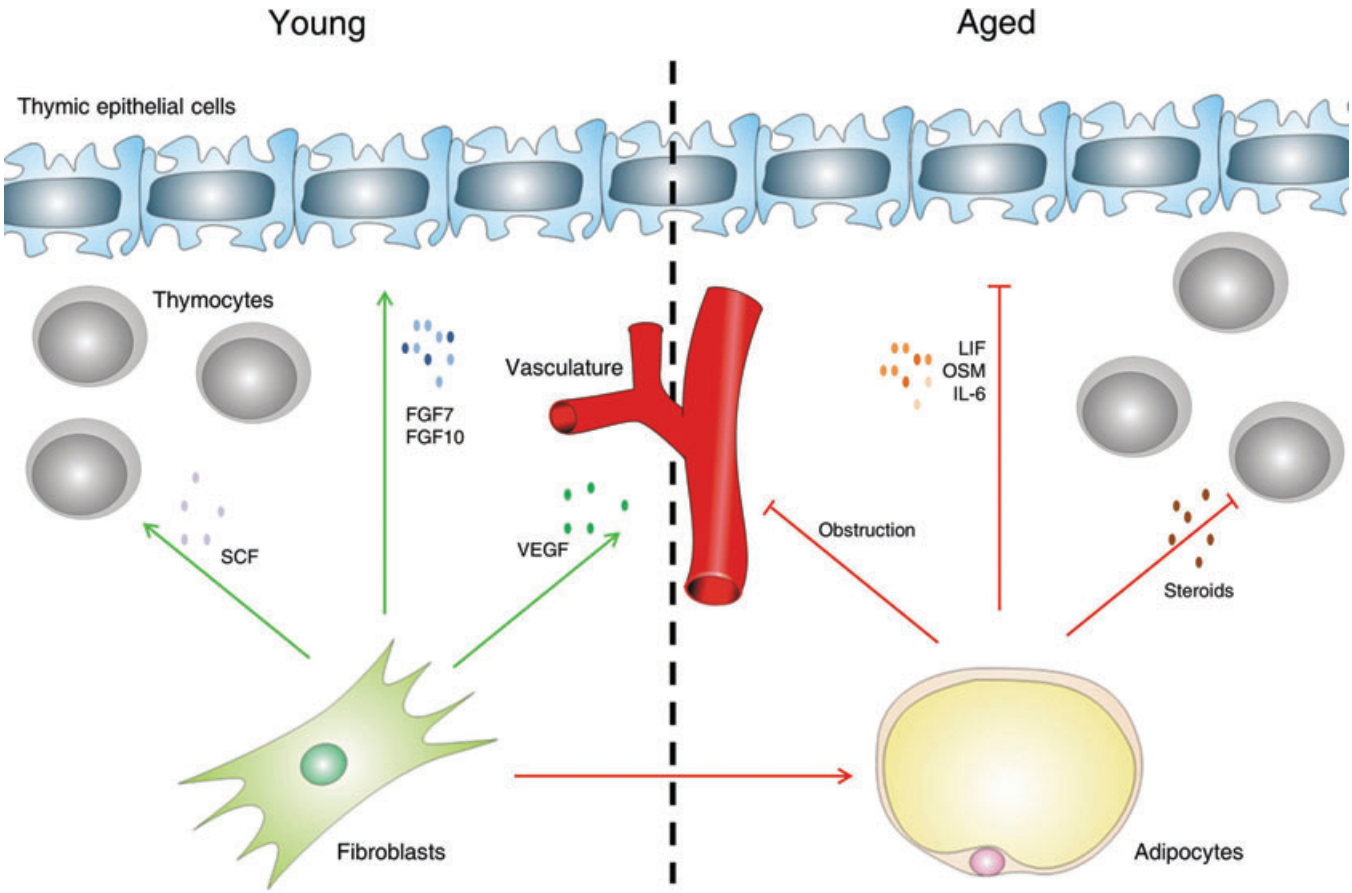

Figure 2. Impact on adipocyte growth on age-related thymic involution. In the young thymus (left), fibroblasts play a rejuvenative function on all the major cellular components of the thymus, stimulating thymocyte proliferation by production of **SCF**, thymic epithelial cell growth by **FGF7**/**FGF10** and vascularization by **VEGF**. In the aged thymus (right), fibroblasts differentiate into adipocytes. As well as losing the rejuvenative function of fibroblasts, the adipocytes have negative effects on the other constituents of the thymus, causing apoptosis of thymocytes by way of steroid production, reducing thymic epithelial cell growth by **LIF**, **OSM**, and **IL**-6 and obstructing the perivascular space of postcapillary venules.

thymus with age and when given exogenously they induce thymic atrophy [37–40]. The local production of this combination of inhibitory factors by adipocytes is likely to transmit a suppressive signal to TECs, which would in turn reduce thymopoiesis and thymic cellularity.

Finally, adipocyte formation within the thymus can lead to anatomical changes. The thymic endothelium is critical for thymic function. It forms a specialized structure at the cortical-medullary junction, which expresses P-selectin, alpha 6 integrins, MECA-79, and VAP-1 [41–43] and allows the import of thymic progenitors from the bone marrow via postcapillary venules (PCVs) [44, 45].The PCVs appear to be double-walled due to the presence of a perivascular space that separates the inner endothelial cell vessel wall from an outer layer of thin epithelial like cells commonly located at the cortical-medullary junction [46–48]. With age, this perivascular space becomes filled by adipocytes, which may limit the function of the PCV in recruiting thymic progenitors [46–48]. As importation of thymic progenitors is an event that is controlled by adhesion molecules, such as P-selectin, whose expression changes periodically (a rate-limiting periodic gated event), a reduction in the thymic progenitors recruited would reduce the final thymic output [43].

Pregnancy-dependent thymic involution

A distinct phenomenon of thymic involution is observed during pregnancy, when the cellularity of the thymus is transiently reduced. Despite this, thymic function remains active, and widespread apoptosis is not observed [49]. This process of thymic involution can be replicated through treatment of either progesterone [50] or estrogens [51], and results in decreased thymocyte proliferation at the DN stage, without induction of apoptosis [50, 52]. While estrogens may contribute to pregnancy-induced thymic involution, progesterone acting directly on TECs is required to drive such involution, as loss of the progesterone receptor from the TEC compartment is sufficient to prevent

pregnancy-associated thymic involution [50]. TECs are therefore required to integrate the hormonal signal and transmit this to DN thymocytes, inducing a slowdown in proliferation rates. An additional contributor to pregnancy-related thymic involution is the decrease in gonadotropin-releasing hormone during pregnancy, as provision of a gonadotropin-releasing hormone agonist during pregnancy partially inhibits involution [53]. It is worth noting that pregnancy also results in a suppression of B-cell differentiation in the bone marrow [54], an effect that can be recapitulated by progesterone and estrogen treatment [55], suggesting that thymic involution during pregnancy is just one of several energy-saving mechanisms initiated by progesterone.

Stress-mediated thymic involution

In addition to the TEC-mediated mechanisms of thymic involution described above, there is a large category of disparate phenomena, which are able to promote thymic atrophy through direct apoptosis of thymocytes. Like TEC-mediated involution, thymocyte-mediated involution results in a thymus of reduced cellularity; however, unlike TEC-mediated involution, where a small "normal" thymus results, thymocyte-mediated involution produces an abnormal thymus, which classically presents with a near-complete loss of DP thymocytes. A number of both infectious and noninfectious stimuli are able to trigger this apoptotic pathway. The injection of lipopolysaccharide (LPS) is sufficient to trigger the same characteristic loss of DP cells and thymic cellularity [4]. Likewise, noninfectious stimuli ranging from stress (both physiological and psychological) [56, 57], fasting [58], inflammation [59], and cancer [60] all cause thymic involution. As discussed above, there are likely to be immunological and/or metabolic advantages to thymic involution during infection; the existence of two discrete infection-sensing pathways (infection-sensing and inflammation-mediated, see "Thymic involution in response to infection") supports the evolutionary importance of involution during infection. It has yet to be established whether there are biological advantages to the involution caused by the noninfectious stimuli that trigger DP loss, or whether these stimuli are the result of a maladaption whereby involution is triggered by the release of mediators that are molecularly similar to those released during infection.

The molecular mediators of many of these stimuli have yet to be characterized; however, it is at least plausible that all share a common molecular pathway, as each is related to the physiological response to stress. Multiple "stress" stimuli feed into the hypothalamic–pituitary–adrenal axis, resulting in the production of glucocorticoids [61, 62]. Adrenalectomy, blocking the production of glucocorticoids, prevents stress-induced thymic involution, demonstrating the necessary role of this pathway [63]. These hormones directly trigger apoptosis of DP thymocytes in an Apaf-1- and caspase-9-dependent manner. The unique susceptibility of DP thymocytes to glucocorticoid-induced apoptosis relates to the lack of expression of Bcl2 within this subset, as loss of Bcl2 at other T cell stages permits glucocorticoid-induced apoptosis. In addition to direct apoptotic effects, glucocorticoids decrease the TCR-signaling capacity, resulting in increased "death by neglect" [63]. Intriguingly, baseline glucocorticoid levels are produced by TECs, but these do not appear to have an essential function for normal thymic development [64, 65], and any homeostatic function that does exist would be overwhelmed by the apoptotic effects of a stress-induced glucocorticoid spike. Additional involution pathways may also be induced by stress stimuli, which can increase the production of IL-6, IL-10, and NGF, and decrease growth hormone and dehydroepiandrosterone, in turn exacerbating thymic involution [66–68].

Concluding remarks

Thymic involution is often thought of as an entropic process. Under this model of thymic biology the thymus, which does not perform essential functions in later life, has not been evolutionarily selected for robustness, and thus is passively worn away by a myriad of microenvironmental fluctuations; however, we believe that this model of thymic involution needs to be reconsidered in light of the recent advances in the understanding of the diversity and complexity of the molecular pathways leading to thymic involution. At least five classes of molecular mediators are capable of driving thymic involution: IFN-α, androgens and estrogens, progesterone, adipose-generated factors, and glucocorticoids. Furthermore, these molecular mediators function through diverse cellular pathways. IFN-α, progesterone, androgens, and estrogens all appear to act directly on TECs. Glucocorticoids, by contrast, act directly on DP thymocytes, and adipocyte-produced factors are likely to function through altering the biology of TECs, endothelial cells, and fibroblasts. Finally, the presence of finely tuned regulation of these pathways, such as the miR-29a-mediated control of the IFN-α-mediated involution pathway, also argues in favor of thymic evolution as an adaptive and directed, rather than entropic, trait. The recent development of new tools to investigate thymic involution suggests that a new research focus on this old topic may be highly informative.

Conflict of Interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: **DP**: double positive · **IFNAR**: interferon α receptor · **MDA-5**: melanoma differentiation-associated gene 5 · **PCVs**: postcapillary venules · **TECs**: thymic epithelial cells

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