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Leukemia Inhibitory Factor, Oncostatin M, IL-6, and Stem Cell Factor mRNA Expression in Human Thymus Increases with Age and Is Associated with Thymic Atrophy[1]

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The roles that thymus cytokines might play in regulating thymic atrophy are not known. Reversing thymic atrophy is important for immune reconstitution in adults. We have studied cytokine mRNA steady-state levels in 45 normal human (aged 3 days to 78 years) and 34 myasthenia gravis thymuses (aged 4 to 75 years) during aging, and correlated cytokine mRNA levels with thymic signal joint (sj) TCR δ excision circle (TREC) levels, a molecular marker for active thymopoiesis. LIF, oncostatin M (OSM), IL-6, M-CSF, and stem cell factor (SCF) mRNA were elevated in normal and myasthenia gravis-aged thymuses, and correlated with decreased levels of thymopoiesis, as determined by either decreased keratin-positive thymic epithelial space or decreased thymic sjTRECs. IL-7 is a key cytokine required during the early stages of thymocyte development. Interestingly, IL-7 mRNA expression did not fall with aging in either normal or myasthenia gravis thymuses. In vivo administration of LIF, OSM, IL-6, or SCF, but not M-CSF, i.p. to mice over 3 days induced thymic atrophy with loss of $CD4^+$, $CD8^+$ cortical thymocytes. Taken together, these data suggest a role for thymic cytokines in the process of thymic atrophy. *The Journal of Immunology,* **2000, 164: 2180–2187.**

During aging, the thymus atrophies and thymic output of T cells falls (1–3). The effect of thymic atrophy on the size of the peripheral T cell pool is minimal, due to the extraordinary capacity of postthymic T cells to proliferate and maintain normal numbers of peripheral T cells (4). However, cancer chemotherapy and AIDS have become common and are associated with loss or damage to the peripheral T cell pool (5).

Recent studies have shown that assay for the presence of TCR δ (*TCRD*) signal joint (sj)[3] TCR excision circles (TRECs) in peripheral blood are markers of either recent thymic emigrants or of long-lived naive T cells that have not divided since emigration from the thymus (6–9). sjTRECs made during rearrangement of the *TCRA* locus result in episomal circles of *TCRD* DNA in newly produced thymocytes. Measurement of sjTRECs in thymocytes of adult subjects has demonstrated ongoing thymopoiesis well into adulthood (10).

The causes of thymic atrophy during aging in humans are not known, but postulated causes are aging of the stem cell population that gives rise to T cells (11, 12), loss of thymic epithelium expressing self-peptides (13), lack of thymocyte rearrangement of *TCRB* genes (14), and aging of the thymic microenvironment with loss of trophic cytokines, such as IL-7 (15–19). Human thymic epithelial cells grown in vitro make a wide variety of hematopoietic cytokines, including G-CSF, M-CSF, GM-CSF, oncostatin M (OSM), LIF, IL-1, IL-6, IL-7, and TGF-α (20–24) (J. S. Sundy, B. F. Haynes, G. D. Sempowski, and D. D. Patel, unpublished observations). In this study we have used *TCRD* sjTREC analysis to quantitate thymopoiesis in normal and myasthenia gravis thymus tissue during aging. In addition, we have utilized RNase protection assays to determine steady-state mRNA levels of 14 key human thymus cytokines during aging, and then determined the effect of in vivo administration in mice of the thymic cytokines that were up-regulated in aged human thymuses. We have found evidence for an active role of LIF, OSM, stem cell factor (SCF), and IL-6 in suppression of thymopoiesis during aging.

Materials and Methods

Human tissues

Normal human thymuses, myasthenia gravis thymuses, and normal adipose tissue were obtained as discarded tissue or after informed consent using a Duke University Institutional Review Board approved protocol. Normal thymus tissues were removed during the course of corrective cardiovascular surgery or other thoracic procedures. Myasthenia gravis thymus tissues were removed during therapeutic thymectomy for treatment of myasthenia gravis. Normal and myasthenia gravis tissue was removed only for clinical or therapeutic reasons, and no tissue was removed solely for research purposes.

Immunohistologic studies

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded 4-μm sections. Tissue sections were deparaffinized with three changes of xylene substitute (Hemo-De, Fisher Scientific, Bay Shore, NY) for 5 min, rinsed in graded ethanols (100%, 100%, and then 95%), then incubated in 0.6% H_2O_2 in absolute methanol (15 min) to block endogenous peroxidase activity. Slides were washed in PBS (pH 7.2) and incubated at room temperature (20 min) with goat or horse serum diluted 1:20 in PBS. Slides were then sequentially incubated with anti-cytokeratin (mixture of AE1/AE3, Boehringer Mannheim, Indianapolis, IN; plus CAM5.2, Becton Dickinson, San Jose, CA; diluted in 1% BSA/PBS) for 45 min, biotinylated secondary Ab for 20 min, and avidin-biotin HRP complexes (VectaStainABC; Vector Laboratories, Burlingame, CA) for 20 min, with

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[3] Abbreviations used in this paper: sj, signal joint; TREC, TCR excision circle; OSM, oncostatin M; SCF, stem cell factor; TES, thymic epithelial space.

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intervening PBS washes. Bound Ab was detected by reactivity with 3,3′-diaminobenzidine plus H_2O_2. Slides were dehydrated in graded ethanols and xylene substitute, then mounted.

The percentage of thymus area consisting of thymic epithelial space (TES) was determined by microscopic examination of hematoxylin and eosin-stained and cytokeratin-immunostained sections using a Zeiss video microscope with grid overlay. TES percentages were obtained for at least six representative fields under 10 × magnification (each field was 1.9 mm^2), which were averaged to obtain the mean % TES for each tissue. TES percentages were determined independently by B.F.H. and L.P.H. and the mean ± SEM of the two sets of data were combined to yield the final %TES ± SEM.

sjTREC analysis

Quantitative-competitive PCR was used to determine levels of *TCRD* locus sjTRECs in whole thymus DNA extracts as previously described by Douek et al. (9). Genomic DNA was extracted from homogenized frozen pieces (~1 cm^3) of normal and myasthenia gravis human thymuses using Trizol (Life Technologies, Gaithersburg, MD), per the manufacturer's protocol. Thymus pieces were weighed before DNA extraction, and the total amount of DNA obtained from each piece was determined by spectrophotometry. The lower limit of detection in the PCR assay was 100 sjTREC molecules per 1 μg of genomic DNA.

RNA isolation and RNase protection assays

Total RNA was isolated from normal and myasthenia gravis thymuses using Trizol (Life Technologies), per the manufacturer's protocol. Approximately 100 mg of tissue was homogenized with an Omni International (Marietta, GA) tissue disrupter in 1 ml of Trizol. Total RNA was extracted according to the manufacturer's protocol, resuspended in diethylpyrocarbonate-treated water, and quantitated by spectrophotometry.

Specific cytokine mRNAs were determined using the multiprobe RiboQuant RNase Protection System (PharMingen, San Diego, CA). Ten micrograms of RNA from each sample was hybridized overnight with ^{32}P-labeled riboprobe sets (PharMingen) and samples processed as described in the manufacturer's instructions. Protected RNA transcripts were separated on a 5% denaturing polyacrylamide gel (National Diagnostics, Atlanta, GA). Cytokine mRNA bands were quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, CA) analysis and reported as % of GAPDH signal.

Protein extraction and cytokine ELISA

Protein was extracted from ~50 mg of snap-frozen thymus and adipose tissue by mincing the tissue in 2 volumes of extraction buffer (1.0% Tween 20, 1 M NaCl, 1× PBS, and 0.1% sodium azide), incubating at 4°C for 1 h, sonicating for 5 min, and then centrifuging at 10,000 × *g* for 10 min. The supernatant was collected and tested for cytokines by a double-determinant ELISA according to the manufacturers' protocols. Human IL-7 was detected with Ab pairs from PharMingen. Human OSM, SCF, LIF, IL-6, and M-CSF were detected with Ab pairs from R&D Systems (Minneapolis, MN). Cytokine protein concentrations in the extracts were determined based on a standard curve of recombinant cytokine provided with the Ab pairs. Whereas OSM, SCF, IL-6, and M-CSF levels were detectable using these methods, LIF levels in thymus and adipose tissue extracts were below the detection range (11 pg/ml) of the ELISA, either due to actual low levels of LIF in tissue or due to inability to extract LIF from tissue.

In vivo cytokine administration

BALB/c mice (female, 8–10 wk old) (The Jackson Laboratory, Bar Harbor, ME) were used to study the in vivo effects of recombinant murine cytokines on thymic atrophy. Mice (n = 3/treatment) were injected i.p. three times daily for 3 days with saline containing 0.1% BSA, or with 2 μg LIF, 2 μg OSM, 2 μg SCF, 3 μg IL-6, or 1 μg M-CSF (0.1% BSA carrier; R&D Systems) using American Association of Laboratory Animal Care guidelines under protocols approved by the Duke University institutional animal use and care committee. One day after the last injection, animals were euthanized and thymuses excised. Percent and absolute numbers of thymocyte subsets were determined by immunofluorescence and flow cytometry using directly labeled mAbs against mouse CD3, CD4, and CD8 (PharMingen) (single cell suspensions of thymocytes were prepared and analyzed as previously described (25)). For double-positive and single-positive thymocyte subsets, flow cytometric histograms of either live

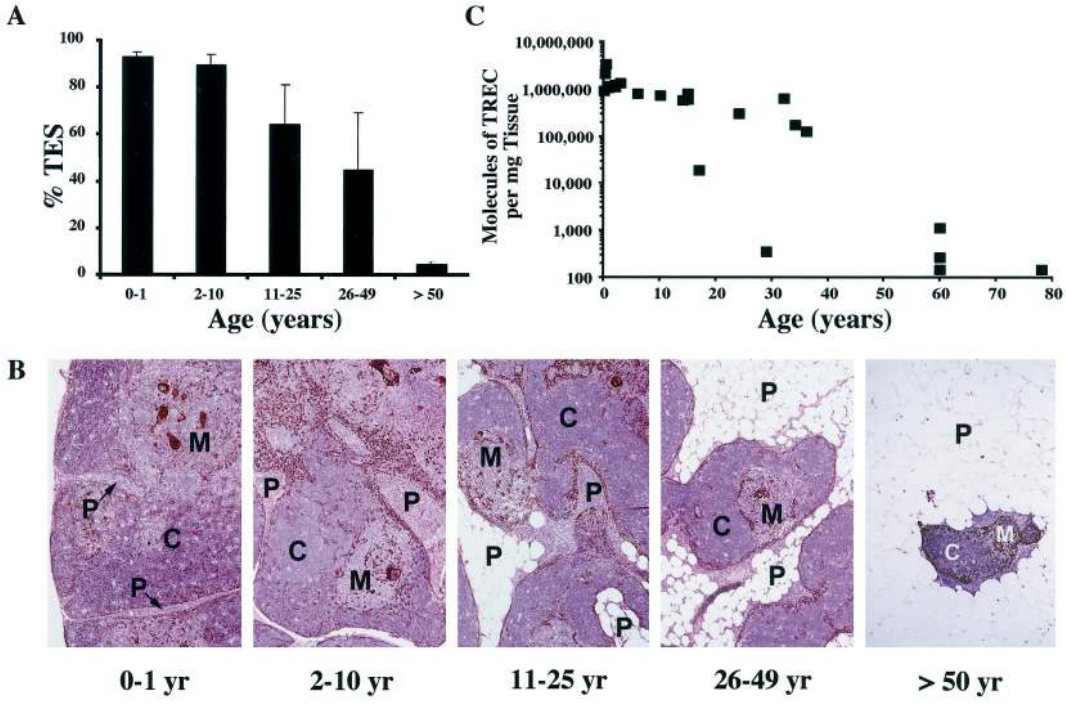

FIGURE 1. TES decreases with age in normal human thymuses. *A*, Percent of the area of the thymus section assessed to be true TES vs age of the thymus. Tissues were grouped into quintiles for data presentation (0–1 year, n = 12; 2–10 years, n = 17; 11–25 years, n = 5; 26–49 years, n = 7; >50 years, n = 4). Significance test of the Pearson correlation of the drop in percent TES vs age showed a strong correlation ($p < 0.000$l). *B,* Representative views of thymus morphology in each age quintile. All thymuses were formalin fixed and paraffin embedded, and sections stained with hematoxylin and eosin (red and blue) and anti-keratin Ab (brown) to determine percent TES (each panel, magnification × 25). Actual age of thymus shown in *B* is 0- to 1-year age group is 3 days; 2- to 10-year age group is 3 years; 11- to 25-year age group is 15 years; 26- to 49-year age group is 36 years; and >50-year age group is 60 years. The figure shows progressive decrease in TES, including cortex (C) and medulla (M), and increase in thymus perivascular space (P) during aging. *C,* Number of molecules of sjTRECs detected per mg wet weight of thymus tissue.

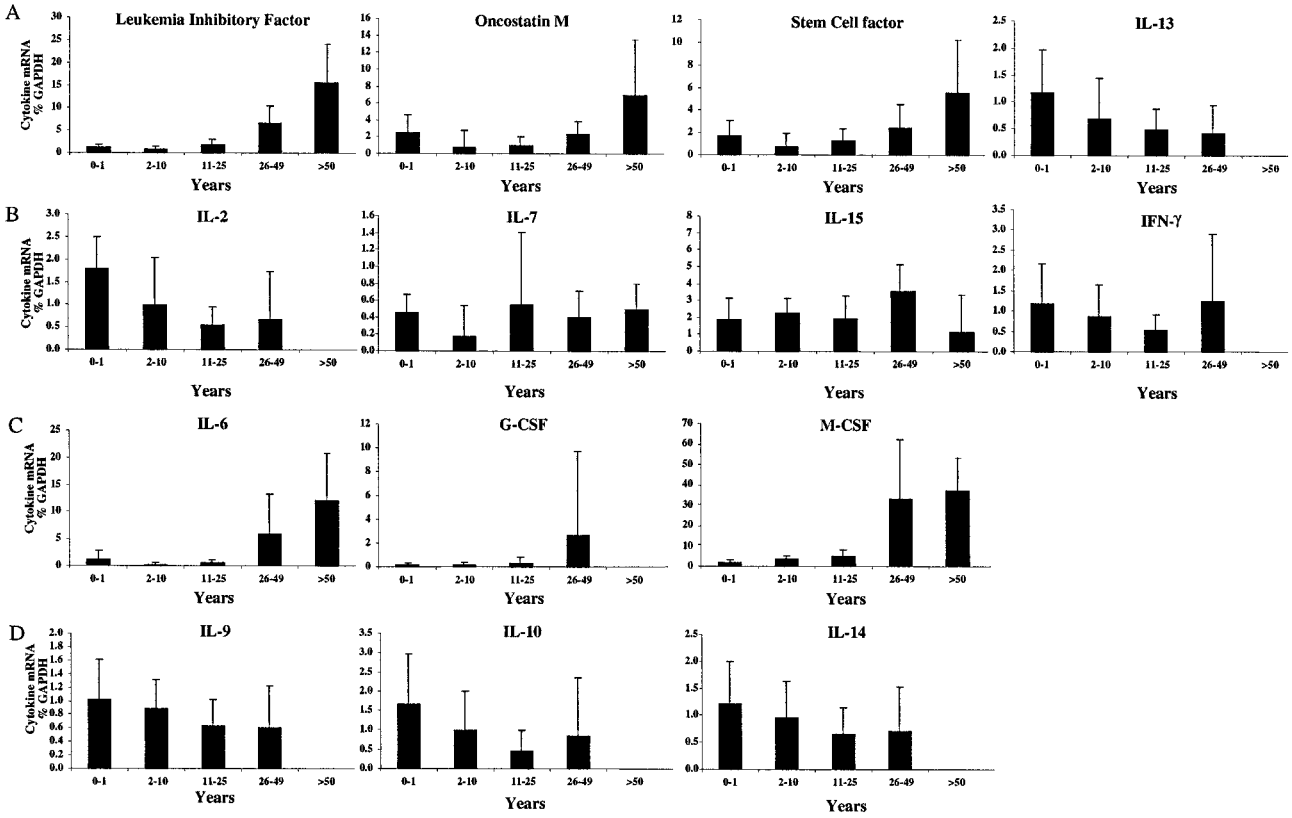

FIGURE 2. Cytokine steady-state mRNA levels in normal thymus tissue from age 3 days through 78 years ($n = 45$). *A–D,* Data are mean cytokine mRNA levels expressed as percent of GADPH mRNA ± SEM. Tissues were grouped into quintiles for data presentation (0–1 year, $n = 12$; 2–10 years, $n = 17$; 11–25 years, $n = 5$; 26–49 years, $n = 7$; >50 years, $n = 4$).

$CD3^+$ thymocytes or total thymocytes were analyzed for CD4 and CD8 expression.

Statistics

Pearson correlation statistical analysis was used to determine the strength and direction of the linear relationship between data sets ($r = -1$ to $+1$). Significance tests were then performed to determine whether r was different from 0. Where indicated Student's t test was used to compare the means for two data sets. Differences were considered significantly different with $p \leq 0.05$.

Results

Analysis of percent TES and sjTRECs in normal human thymus during aging

We studied 45 normal human thymuses from individuals 3 days to 78 years old and divided the thymuses into five age groups (Fig. 1). Hematoxylin and eosin staining and anti-keratin immunohistochemistry were utilized to distinguish the TES (keratin-positive) from the keratin-negative thymic perivascular space. In normal thymuses, the percent of the whole thymus that contained the TES (cortex and medulla) fell to 5% or less by age 78 ($p < 0.0001$) (Fig. 1*A*). Fig. 1*B* shows representative hematoxylin and eosin plus anti-keratin-labeled thymus tissue sections from each age group studied and demonstrates a progressive decrease in TES and concurrent increase in thymus perivascular space during aging.

To quantitate the level of thymopoiesis in these normal human thymus tissues, we assayed for the presence of sjTRECs. We found sjTRECs per mg whole thymus tissue decreased with increasing age ($p < 0.001$), as well as with decreased percent TES ($p < 0.0001$) (Fig. 1*C*). Thymocyte cell suspensions were available for sjTREC analysis from seven thymuses studied (ages 2–34 years). sjTREC levels per 10^5 thymocytes did not fall in these samples, indicating that on a per thymocyte basis TCR rearrangement continued into adulthood (data not shown).

mRNA expression of thymic cytokines during aging

We next studied the mRNA expression profile of 14 cytokines in normal thymuses from 50 individuals during aging using a multiplex RNase protection assay (Fig. 2). We analyzed the cytokines in

Table I. *Correlation of cytokine mRNA levels in normal thymus tissue with age and molecules of TRECs*[a]

Cytokine	Cytokine mRNA Level vs Age		Cytokine mRNA Level vs Decreasing sjTREC	
	Change	p value[b]	Change	p value[b]
LIF	↑	0.01	↑	0.01
OSM	↑	0.01	NC	0.07
SCF	↑	0.01	↑	0.05
IL-13	↓	0.01	NC	0.20
IL-2	↓	0.01	↓	0.01
IL-7	NC	0.26	NC	0.67
IL-15	NC	0.72	NC	0.76
IFN-γ	NC	0.17	NC	0.20
IL-6	↑	0.01	↑	0.03
G-CSF	NC	0.34	NC	0.86
M-CSF	↑	0.01	↑	0.02
IL-9	↓	0.01	NC	0.18
IL-10	↓	0.03	NC	0.15
IL-14	↓	0.01	NC	0.22

[a] NC, no change; ↑, increase with variable; ↓, decrease with variable.

[b] The p values were determined by significance test of the Pearson correlation coefficient. Changes were assumed to be significant with $p \leq 0.05$, comparing data from patients in quintile 5 (>50 years old) vs data from patients in quintile 1 (0–1 years old).

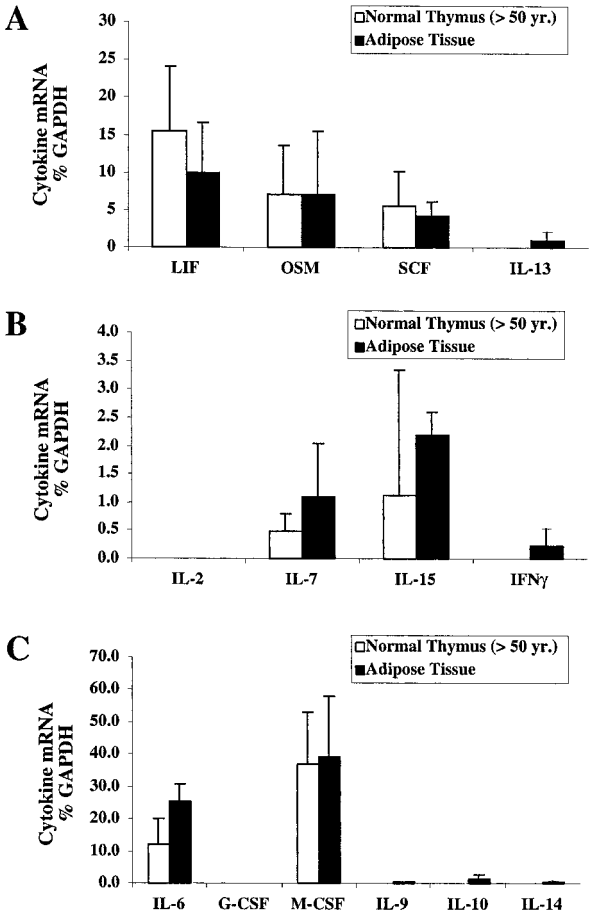

FIGURE 3. Cytokine mRNA expression profiles in aged thymus tissue are similar to those of normal adipose tissue. *A–C,* Steady-state mRNA levels in adipose vs normal thymus tissue. Adipose tissue from multiple sites (mediastinal perithymic area, $n = 2$; perirenal area, $n = 2$; and subcutaneous abdomen, $n = 1$; total, $n = 5$) were assayed for steady-state mRNA levels by RNase protection assays as in Fig. 2, and compared with data from normal thymuses >50 years of age ($n = 4$). Data are expressed as cytokine mRNA as a percent of GADPH mRNA ± SEM.

four groups: LIF, OSM, SCF, and IL-13 (Fig. 2*A*); IL-2, IL-7, IL-15, and IFN-γ (Fig. 2*B*); IL-6, G-CSF, and M-CSF (Fig. 2*C*); and IL-9, IL-10, and IL-14 (Fig. 2*D*).

We found that LIF, OSM, SCF, IL-6, and M-CSF steady-state mRNAs were expressed significantly higher in aged human thymus (Fig. 2, Table I). The increase in steady-state mRNA expression of LIF, SCF, IL-6, and M-CSF correlated with age and also correlated with decreasing thymic sjTRECs (Table I). Increased levels of mRNA for OSM correlated with increasing age, as well as decreasing percent TES ($p < 0.001$), and approached significance ($p < 0.07$) with decreasing sjTRECs (Table I).

Five cytokines that were expressed in the young thymuses when thymopoiesis is greatest (IL-2, IL-9, IL-10, IL-13, and IL-14) ceased to be expressed in aged thymuses (Table I). Expression of IL-2 mRNA significantly decreased with age and decreasing sjTRECs (Table I). IL-9, IL-10, IL-13, and IL-14 decreased with age, but not significantly with decreasing sjTREC levels (Table I). Interestingly, no change in steady-state mRNA levels was seen for IL-7, IL-15, IFN-γ, or G-CSF with respect to age or number of sjTRECs (Table I).

To determine whether the expanded adipose tissue of the thymic perivascular space (Fig. 1*B*) could be in part responsible for elevated cytokine mRNA in normal aged human thymuses, we obtained adipose tissue from multiple sites (mediastinal perithymic area, $n = 2$; perirenal, $n = 2$; and subcutaneous, $n = 1$; total, $n = 5$). RNase protection assays were performed on total RNA isolated from adipose samples and compared with the cytokine mRNA level obtained from normal thymuses >50 years old (Fig. 3). Interestingly, we saw that the cytokine mRNA profile for adipose tissue was similar to that seen in aged normal thymus (Fig. 3). LIF, OSM, SCF, IL-7, IL-15, IL-6, and M-CSF mRNA were all expressed in adipose tissue in levels comparable to aged thymus. To confirm that protein could be measured in tissue that had high expression of cytokine mRNAs by RNase protection, we assayed for cytokine protein in adipose and >50 years thymus tissue extracts by ELISA. We found cytokine protein present for OSM, SCF, IL-6, M-CSF, and IL-7 in both adipose and aged thymus tissue (data not shown). LIF protein levels in these tissues were either below detection in our assay, or LIF protein was not extracted optimally with the methods used.

Analysis of percent TES and sjTRECs in myasthenia gravis thymus during aging

Myasthenia gravis is an autoimmune disease characterized by neuromuscular weakness and is associated with thymoma in 10% of patients (26, 27). Myasthenia gravis thymuses are frequently characterized histologically as hyperplastic for age, with lymphoid areas containing B cell germinal centers (26, 27). It has recently been realized that hyperplastic lymphoid areas of the myasthenic thymus are entirely in the thymic perivascular space, whereas actual thymopoiesis is decreased in the TES (26–28). To confirm observations of ongoing thymopoiesis in adults, we compared percent TES and sjTREC levels in a cohort of 34 myasthenia gravis thymuses (age 4–75 years). Our data demonstrated that as the myasthenic thymus aged, TES also fell to 5% or less by age 75 (Fig. 4*A*) ($p < 0.0001$) and correlated with decreasing thymus sjTREC levels ($p < 0.0001$) (Fig. 4*B*).

To confirm our observations of up-regulated production of specific cytokines in aged normal thymuses, we next determined the mRNA expression profile of thymic cytokines in myasthenia gravis thymuses (Fig. 5, *A–D*). We found that LIF, OSM, SCF, IL-13, IL-7, IFN-γ, IL-6, and M-CSF steady-state mRNAs were expressed significantly higher in aged myasthenia gravis thymuses

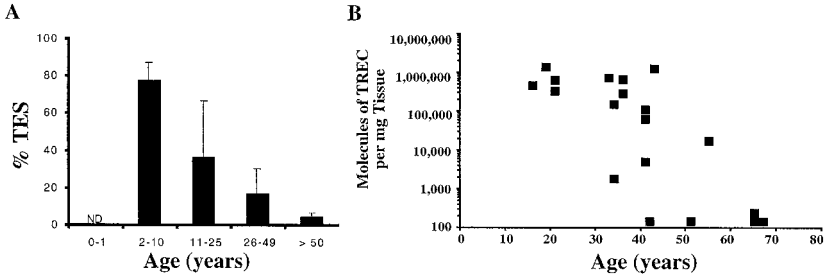

FIGURE 4. TES decreases with age in myasthenia gravis thymuses. *A,* Percent of the area of the thymus section assessed to be true TES vs age of the thymus. Tissues were grouped into quintiles for data presentation (0–1 year, none available; 2–10 years, $n = 2$; 11–25 years, $n = 8$; 26–49 years, $n = 14$; >50 years, $n = 10$). *B,* Number of molecules of sjTREC detected per mg wet weight of myasthenic thymuses (age 16–67 years, $n = 20$). ND, not done.

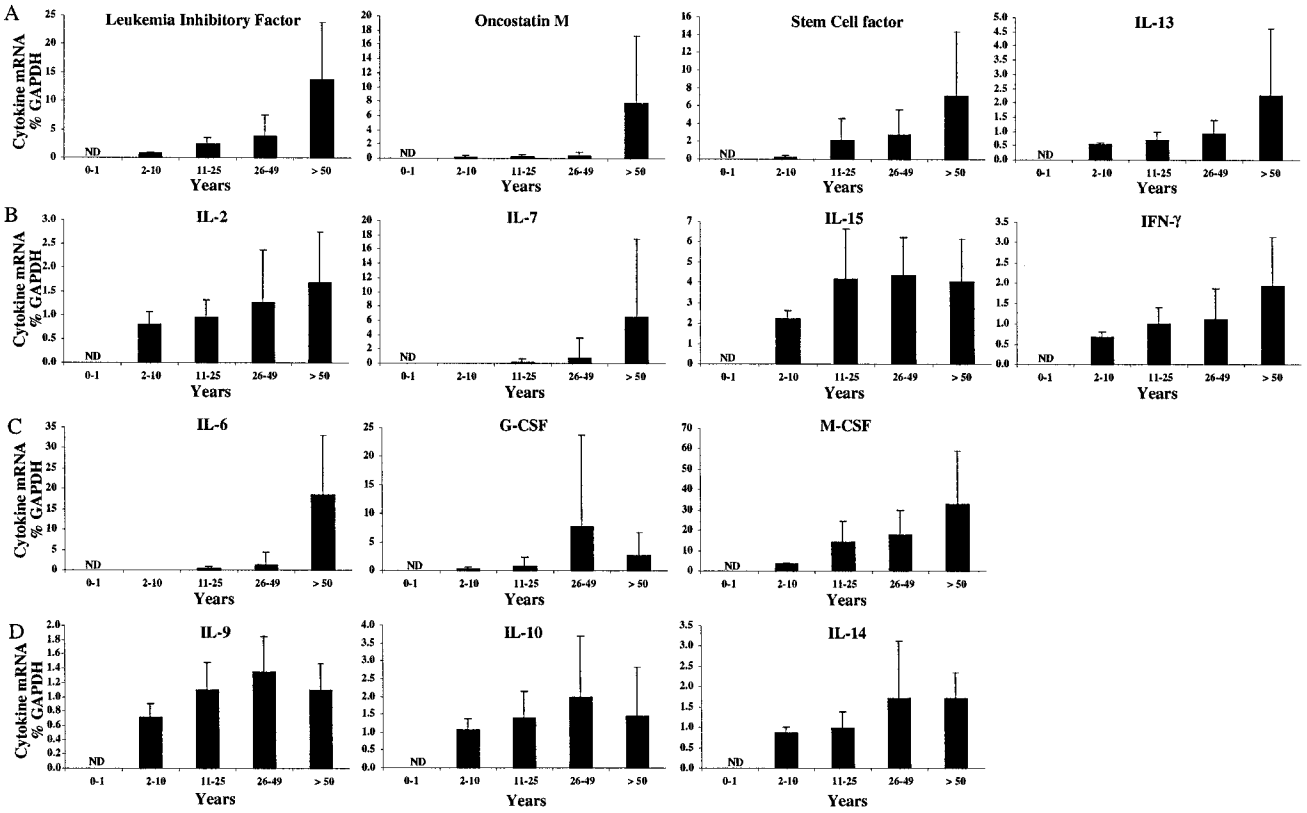

FIGURE 5. Cytokine steady-state mRNA levels in myasthenia gravis thymus tissue from 4 through 75 years of age ($n = 23$). *A–D,* Data are mean cytokine mRNA levels expressed as percent of GADPH mRNA ± SEM. ND, not done. Tissues were grouped into quintiles for data presentation (0–1 year, none available; 2–10 years, $n = 2$; 11–25 years, $n = 8$; 26–49 years, $n = 14$; >50 years, $n = 10$).

compared with younger myasthenia gravis thymuses (Table II). Increased levels of mRNA for LIF, SCF, and M-CSF correlated with decreasing sjTRECs (Table II). All of the other cytokine mRNAs analyzed remained unchanged with increasing age in myasthenia gravis thymuses (Table II). A striking observation in myasthenia gravis thymuses was that the cytokine mRNAs that were unchanged (IL-7 and IFN-γ) during aging in normal thymuses were significantly elevated in aged myasthenia gravis thymuses, and those cytokine mRNAs that fell during normal aging (IL-2, IL-9, IL-10, IL-13, and IL-4) did not fall in myasthenia gravis thymuses (Table III). In particular, it is important to note that in myasthenia gravis thymuses, IL-7 mRNA expression increased with age despite marked atrophy of TES and fall in thymus sjTREC levels. Thus, the presence of expression of IL-7 and other

Table II. *Correlation of cytokine mRNA levels in myasthenia gravis thymus tissue with age and molecules of TRECs*[a]

	Cytokine mRNA Level vs Age		Cytokine mRNA Level vs Decreasing sjTREC	
Cytokine	Change	*p* value[b]	Change	*p* value[b]
LIF	↑	0.01	↑	0.02
OSM	↑	0.01	NC	0.21
SCF	↑	0.01	↑	0.02
IL-13	↑	0.01	NC	0.18
IL-2	NC	0.08	NC	0.36
IL-7	↑	0.03	NC	0.35
IL-15	NC	0.38	NC	0.35
IFN-γ	↑	0.01	NC	0.10
IL-6	↑	0.01	NC	0.07
G-CSF	NC	0.43	NC	0.24
M-CSF	↑	0.01	↑	0.01
IL-9	NC	0.34	NC	0.47
IL-10	NC	0.59	NC	0.29
IL-14	NC	0.12	NC	0.19

[a] NC, no change; ↑, increase with variable.
[b] The *p* values were determined by significance test of the Pearson correlation coefficient. Changes were assumed to be significant with $p \leq 0.05$, comparing data from patients in quintile 5 (>50 years old) vs data from patients in quintile 2 (2–10 years old).

Table III. *Comparison of changes in thymic cytokine steady-state mRNA levels during aging in normal versus myasthenia gravis thymuses*[a]

	Cytokine Level vs Age	
Cytokine	Normal	MG
LIF	↑	↑
OSM	↑	↑
SCF	↑	↑
IL-13	↓	↑[b]
IL-2	↓	NC[b]
IL-7	NC	↑
IL-15	NC	NC
IFN-γ	NC	↑[b]
IL-6	↑	↑
G-CSF	NC	NC
M-CSF	↑	↑
IL-9	↓	NC[b]
IL-10	↓	NC[b]
IL-14	↓	NC[b]

[a] NC, no change; ↑, increase with variable; ↓, decrease with variable.
[b] Significant difference between normal and myasthenia gravis (MG) thymuses in >50-year age group. The *p* values were determined by Student's *t* test. Changes were significant with $p \leq 0.05$.

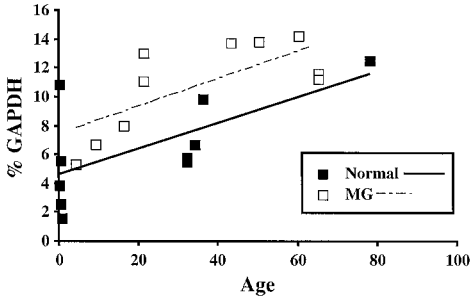

FIGURE 6. TGF-β steady-state mRNA levels in normal and myasthenia gravis (MG) thymus tissue. Ten normal thymus tissues ages 0–78 years and 10 myasthenia gravis thymus tissues ages 4–65 years were analyzed by RNase protection assay, and data are shown as percent GAPDH signal. There were significant increases in TGF-β1 steady-state mRNA levels with age in both normal ($p < 0.043$) and myasthenia gravis ($p < 0.019$) thymuses. Data were analyzed by Pearson correlation statistical analysis.

trophic cytokines in myasthenic thymuses in the face of decreased thymopoiesis suggested the possibility of an active process of suppression of thymopoiesis during aging.

Analysis of TGF-β mRNA in normal and myasthenia gravis thymus tissue

TGF-β is known to be expressed by the thymic microenvironment and has been demonstrated to be an inhibitor of T cell proliferation and play a role in murine T cell maturation (29–31). In addition, TGF-β1 has been shown to synergize with epidermal growth factor for up-regulation of LIF and IL-6 mRNA levels (46). To explore the potential role of TGF-β in mediating age-related atrophy in normal and myasthenia gravis thymus tissue, we determined the level of TGF-β mRNA in 10 normal and 10 myasthenia gravis thymus tissues representative of 0–78 years and 4–65 years of age, respectively (Fig. 6). Our analysis by RNase protection assay revealed detectable levels of the TGF-β1 isoform. In both normal and myasthenia gravis thymus tissue there was a correlation between increasing TGF-β1 mRNA levels and increasing age ($p < 0.043$ and $p < 0.019$, respectively) (Fig. 6). The level of TGF-β1 mRNA detected in myasthenia gravis thymus tissue was not significantly higher than that in normal thymus tissue ($p < 0.078$). We were unable to detect TGF-β2 and TGF-β3 mRNA transcripts in total RNA from whole thymus tissue with this technique. However, studies utilizing RT-PCR have demonstrated detectable levels of TGF-β2 in whole thymus tissue (data not shown). Taken together, these data suggest that TGF-β1 may also play a role in thymic atrophy directly, or indirectly by up-regulating thymosuppressive cytokines, such as IL-6 and LIF (46).

Table IV. *Administration of recombinanat LIF, OSM, SCF, or IL-6 to mice induces thymic atrophy*

Treatment[a]	Total Thymus Weight (mg)	Total Thymocytes
Saline ($n = 6$)	45 ± 10	37±17
LIF, 2 μg ($n = 6$)	15 ± 3[b]	7 ± 6[b]
OSM, 2 μg ($n = 6$)	14 ± 4[b]	4 ± 3[b]
SCF, 2 μg ($n = 6$)	35 ± 7	17 ± 11[b]
IL-6, 3 μg ($n = 6$)	31 ± 5[b]	16 ± 10[b]
M-CSF, 1 μg ($n = 6$)	37 ± 11	29 ± 11

[a] All injections were i.p. three times daily for 3 days. Shown are combined data from two independent experiments ($n = 3$ mice per group per experiment).

[b] Significant difference between cytokine-treated and saline-injected control mice. The p values were determined by Student's t test. Changes were significant with $p \leq 0.05$.

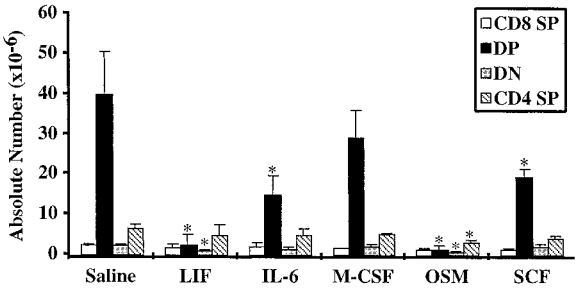

FIGURE 7. In vivo administration of recombinant murine cytokines induced thymic atrophy in BALB/c mice (female, 8–10 wk old). Mice ($n = 3$/treatment) were injected i.p. three times daily for 3 days with saline containing 0.1% BSA, or with 2 μg LIF, 2 μg OSM, 2 μg SCF, 3 μg IL-6, or 1 μg M-CSF. Percent and absolute numbers of thymocyte subsets were determined by immunofluorescence and flow cytometry using directly conjugated mAbs against mouse CD3, CD4, and CD8. For double-positive and single-positive thymocyte subsets, flow cytometric histograms of live $CD3^+$ thymocytes were analyzed for CD4 and CD8 expression. Data are expressed as absolute mean ± SEM. Representative experiment of two performed. SP, single positive; DP, double positive; DN, double negative. *, $p \leq 0.05$ (Student's t test) compared with saline-injected control mice.

Effect of administration of LIF, OSM, IL-6, SCF, and M-CSF on thymus size in mice

To test the hypothesis that the up-regulated thymic cytokines in atrophic, aged human thymuses might inhibit thymopoiesis, we administered LIF, OSM, SCF, IL-6, or M-CSF i.p. into BALB/c mice and determined whether any of these cytokines could induce acute thymic atrophy in vivo. We found that injection of LIF, OSM, SCF, and IL-6 three times a day for 3 days, in the dose regimens listed in Table IV, induced thymic atrophy when compared with a saline control. Total thymus weights and thymocyte numbers were significantly decreased in LIF-, OSM-, and IL-6-treated mice, whereas thymocyte numbers were significantly decreased in SCF-treated mice (Table IV). Phenotypic analysis of thymocytes obtained from each group of mice demonstrated that LIF-, OSM-, and SCF-treated mice all had significantly decreased absolute numbers of $CD3^-CD4^-CD8^-$ (triple negative), $CD3^+CD4^+CD8^+$ (triple positive), as well as $CD4^+$ and $CD8^+$ single-positive thymocytes (Fig. 7). In one experiment shown in Fig. 7 ($n = 3$ mice per group) IL-6 induced a decrease in absolute numbers of $CD3^+CD4^+CD8^+$ ($5.8 \times 10^6 \pm 2.6 \times 10^6$ cells vs saline control, 14.6×10^6 cells $\pm 4.7 \times 10^6$) and just missed statistical significance ($p = 0.08$). In a second set of experiments ($n = 3$ mice per group) the results with LIF, OSM, and SCF were similar, and IL-6 induced a significant loss ($p = 0.04$) of $CD4^+CD8^+$ (double positive) thymocytes ($14.8 \times 10^6 \pm 4.8 \times 10^6$ cells vs saline control, 40.0×10^6 cells $\pm 10.5 \times 10^6$).

Discussion

In this study we have shown for the first time up-regulation of LIF, OSM, SCF, M-CSF, and IL-6 mRNA in normal atrophic human thymuses in the face of continued expression of the key thymotrophic cytokine, IL-7. We also studied thymic cytokine expression in myasthenia gravis thymus tissue during aging and found similar up-regulated thymic LIF, OSM, IL-6, SCF, and M-CSF, as well as IL-7 expression during aging. Interestingly, in contrast to normal atrophic thymuses, myasthenia gravis atrophic thymuses did not have decreased expression of IL-2, IL-9, IL-10, IL-13, or IL-14 cytokine mRNA, demonstrating that thymic atrophy occurs in the presence of expression of these cytokines as well. The observation that thymic atrophy occurred in the setting of continued thymic

expression of IL-7 and other trophic cytokines suggested the hypothesis that active mechanisms of suppression of thymopoiesis might be operative during the process of thymus atrophy. Treatment of mice with LIF, OSM, SCF, and to a lesser degree IL-6 induced thymic atrophy in vivo.

It is of interest that M-CSF mRNA levels were significantly elevated in both normal and myasthenia gravis-aged thymus tissue. However, administration of M-CSF alone failed to induce thymic atrophy in BALB/c mice. There are several possibilities for these observations. First, M-CSF may be related to human thymic atrophy but not to murine thymic atrophy, i.e., there may be species differences. Second, M-CSF alone may not be sufficient to cause thymic atrophy but may function in combination with other cytokines such as LIF, OSM, SCF, or IL-6. Studies to determine the effect of combinations of cytokines in murine thymic atrophy will directly address this issue.

Studies of transgenic mice that overexpress LIF, OSM, or SCF have suggested that dysregulated thymic expression of these cytokines may be associated with thymic abnormalities. LIF and OSM transgenic mice had decreased $CD4^+CD8^+$ double-positive thymocytes and numerous thymic B cell follicles (32, 33). Injection of mice by others with LIF in the same regimen used in our study also induced thymic atrophy in mice (34). Our study shows similar effects of injected LIF, as well as for the first time shows the effects of OSM, SCF, and IL-6 on thymic atrophy. Although studies in mice are difficult to extrapolate to humans, our results do show an active acute thymopoietic suppressive effect in mice of these cytokines. However, the relevance of our observations in mice to the pathogenesis of human thymic atrophy in aging remains to be shown.

The role of testosterone in active suppression of thymopoiesis and induction of thymic atrophy has been demonstrated by reversal of thymic atrophy by chemical castration by administration of luteinizing hormone releasing hormone or by surgical castration (35). Clearly, corticosteroids also actively suppress thymopoiesis and induce the selective loss of cortical thymocytes (36). One interesting relationship between LIF overproduction and glucocorticoid regulation comes from the observation in LIF deficient animals of decreased adrenocorticotropin (ACTH) secretion (37). It has been demonstrated that LIF is a potent stimulus of pituitary ACTH secretion, modulating the hypothalamic-pituitary-adrenal axis in response to various inflammatory stimuli (38). It will be of great interest to study the levels of glucocorticoids and testosterone in LIF-, OSM-, SCF-, and IL-6-treated mice and in aging humans, both in plasma and in thymus tissue. Thus, it is a plausible notion that LIF may act indirectly through induction of corticosteroids or other factors to suppress thymopoiesis.

Several factors have been reported to be trophic for thymopoiesis, including IL-1 and GM-CSF (39), IL-7 (18, 19, 40), human growth hormone (41), insulin like growth factor-1 (41), and IL-11 plus IL-3 (42). Mutations in IL-7, IL-7Rα, or in the common γ chain (γ_c) of the IL-2, IL-7, and IL-15 receptors leads to SCID (43–45). The SCID syndrome of IL-7-deficient mice can be reversed by bcl-2, demonstrating that IL-7 delivers anti-apoptotic signals to developing thymocytes (44). Thus, as the thymus ages, up-regulated production of suppressive cytokines (such as LIF, OSM, SCF, and IL-6) by thymic epithelial cells, adipocytes, or other stromal cells may directly or indirectly through other factors such as corticosteroids overcome anti-apoptotic signals of trophic cytokines such as IL-7, and thus may play a role in contributing to thymic atrophy. Alternatively, as the thymus atrophies, IL-7 and other trophic cytokine production may not be located in the correct thymic compartment for thymopoiesis to proceed normally (i.e., IL-7 may be in the perivascular space and not the TES). Studies are underway to directly address these issues.

In general, myasthenia gravis patients in this study received either no medications or various doses of mestinon, prednisone, or rarely azathioprine. Thus, we cannot rule out an effect of medications on the cytokine profiles seen in myasthenia gravis thymus tissue. However, the importance of the myasthenia gravis data set lies in the observation of continued expression of IL-7 during thymic atrophy that occurs in myasthenia gravis.

Finally, our study shows for the first time the rate of decline of sjTREC levels in whole thymus tissue with aging. We show measurable sjTRECs up to age 60, and importantly determined sjTRECs per mg of thymus tissue. Jamieson et al. (10) showed that up to age 50, sjTRECs per 10^5 thymocytes are high, indicating that thymocyte function during aging is constant on a per thymocyte basis, whereas our data directly demonstrate that the total number of functionally rearranged thymocytes per whole thymus declines with age.

Thus, for patients with HIV infection, and for cancer patients undergoing chemotherapy or bone-marrow transplantation, the critical question is what therapeutic strategies could be employed to promote reconstitution of T cells by enhancing thymopoiesis. Our study demonstrates up-regulated expression of mRNA of cytokines capable of suppressing thymopoiesis (LIF, OSM, IL-6, and SCF) in the atrophic thymus. If a pathophysiologic role in human thymic atrophy for these up-regulated suppressive cytokines can be shown, then these data raise the possibility of treating AIDS and chemotherapy treatment patients with a combination of trophic cytokines and inhibitors of suppressive cytokines to improve postnatal thymic function.

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