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Age-associated alterations in the levels of cytotoxic lipid molecular species and oxidative stress in the murine thymus are reduced by growth hormone treatment

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Abstract

During age-associated thymic involution, thymocytes decrease and lipid-laden cells accumulate. However, if and how aging affects the thymic lipid profile is not well understood, nor is it known if the hormonal milieu modifies this process. Here we demonstrate a correlation between reduced thymocyte numbers and markers of inflammation and oxidative stress with age. Evaluating the lipidomics profile of the whole thymus, between the ages of 4 (young) and 18 months (old), we found increased amounts of triacylglycerides, free cholesterol, cholesterol ester and 4-hydroxynonenal (4-HNE) with age. Moreover, levels of C24:0 and C24:1 sphingomyelins and ceramide C16:0 were elevated in 12–14 month-old (middle-aged) mice while the levels of sulfatide ceramide and ganglioside GD1a increased in the old thymus. Evaluating isolated thymocytes, we found increased levels of cholesterol ester and 4-HNE adducts, as compared to young mice. Next, we treated middle-aged mice with growth hormone (GH), which has been considered a potent immunomodulator. GH reduced thymic levels of TNF- α and 4-HNE and increased the number of thymocytes as well as the thymic levels of dihydroceramide, a ceramide

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Author contribution

DDT, MPM, RGC and VMC participated in the conception and design of the experiments; AB, AT, DDT, MPM, RGC and VMC analyzed the data; DDT, MPM, RGC and VMC interpreted the data and wrote the paper.

precursor and autophagic stimuli for cell survival. In conclusion, GH treatment attenuated inflammation and age-related increases in oxidative stress and lipotoxicity in the thymus.

Keywords

aging; growth hormone; lipids; pro-inflammatory factors; oxidative stress; thymus

1. Introduction

During aging, there occurs an increase in the amount of reactive oxygen species (ROS)-mediated damage to major cellular macromolecules including proteins, nucleic acids and lipids (Ames *et al.*, 1993; Berlett and Stadtman, 1997). Oxidative stress promotes an increase in the circulating levels of pro-inflammatory cytokines (Franceschi *et al.*, 2000), which stimulates further ROS generation and the production of cytotoxic lipid metabolites including aldehydes such as 4-hydroxynonenal (4-HNE) and long chain ceramides (Hannun & Obeid, 2002; Cutler *et al.*, 2004; Mattson, 2009). Oxidative stress is also associated with dyslipidemia during aging (Kirkland *et al.* 2002). In this context, the redistribution and accumulation of various species of lipids and lipid droplets occurs not only in blood vessels but also in lymphohematopoietic organs, including the thymus (Steinmann *et al.* 1985; Flores *et al.*, 1999; Mello-Coelho *et al.* 2010; Langhi *et al.* 2015).

The thymus is an organ that diminishes its capacity to generate immunocompetent T cells with aging (Douek *et al.* 1997; Taub and Longo, 2005; Taub *et al.* 2010). The age-related decline of thymic function is inversely correlated with an increase of intrathymic lipid-laden multilocular and adipose cells, which produce pro-inflammatory molecules, such as leukemia inhibitory factor (LIF), interleukin-6 (IL-6) and other ligands of the gp130 receptor family (Sempowsky *et al.* 2000; de Mello-Coelho *et al.* 2004; Taub *et al.* 2010). Moreover, perithymic and perivascular lipid-laden cells (LLC) express CCR5 and pro-inflammatory chemokine ligands to CCR5 increase in the aging thymus, suggesting that intrathymic LLC could be, originally, preadipocytes migrating from the perithymic adipose tissue into the thymic microenvironment (de Mello-Coelho *et al.* 2010). Conjointly, these factors might contribute to oxidative stress and thymocyte death. In addition, age-related thymic involution is associated with decreased lymphocyte differentiation, and defects in the stromal cell niche (Ortman *et al.* 2002; Gui *et al.* 2007), including thymic epithelial cells, which are responsible for the production of several hormones, growth factors and cytokines that regulate thymocyte maturation and survival (Boyd *et al.* 1993; de Mello-Coelho *et al.* 1998; Taub *et al.* 2010).

Growth hormone (GH) has been considered as a treatment to counteract many age-related degenerative processes (Hartmann *et al.* 2013) and as a potential immunoregulator. In this context, human GH (hGH) treatment stimulated thymopoiesis and T lymphocyte function in human immunodeficiency conditions in middle-aged individuals (Napolitano *et al.* 2008; Herasimtschuk *et al.*, 2013). Human GH, which binds to GH receptor and prolactin receptor, increased the lymphoid and hematopoietic compartments in rat thymus and bone marrow, respectively. Systemic treatment with ovine GH, which binds specifically to GH receptor,

also enhanced the size and function of the mouse thymus (Murphy *et al.* 1992). Kelley *et al.* (1986) using implant of GH3 rat pituitary adenoma cell line, which secretes GH and prolactin, showed an increase in cortical thymocytes associated with decreased fat locules in the thymic parenchyma. Moreover, GH also decreased the density of bone marrow adipocytes in aged rats (French *et al.*, 2002). Furthermore, we showed that ovine GH promoted an increase in the output of thymic T cells and their migration to secondary lymphoid organs in mice (Smaniotto *et al.* 2004; 2005). Later, Napolitano *et al.* (2008) found that treatment of adult HIV-infected individuals with hGH increased the number of cells with T cell receptor excision circle (TREC) in the peripheral blood, indicating an stimulatory effect of GH in thymic T cell differentiation and output. However, the relationships among the effects of GH on thymopoiesis, oxidative stress and lipid metabolism in the aging thymus are still unclear. In the current study, we describe several age-related alterations of lipid metabolism and oxidative stress status that occur in the mouse thymus and in isolated thymocytes. In addition, we found that GH administration in middle-aged male mice was able to reverse age-related oxidative stress, pro-inflammatory processes and lipotoxicity in the thymus.

2. Material and Methods

2.1. Mice

Male C57BL/6 mice between 2 and 24 months of age were used for in vivo experiments, and cell culture experiments utilized thymocytes from young male BALB/c mice. Mouse colonies were maintained in the National Institute on Aging vivarium where they were housed in environmentally controlled rooms with a 12 hour light-dark cycle in agreement with the procedures outlined in the “Guide for the Care and Use of Laboratory Animals” [NIH publication no.86-23, 1985]. All procedures were approved by the Animal Care and Use Committee of the National Institute on Aging Intramural Research Program.

2.2. Growth hormone *in vivo* treatment

Mice at 12–14 or 16 months of age were anesthetized with an i.p. injection of anesthetic (10 mg ketamine)/ 1 mg xylazine/mL; 0.1 mL/10 g body weight]. In sterile conditions, a small incision was made in the dorsal neck area of the mice and a mini-osmotic pump (ALZET, Cupertino, CA) containing ovine GH (40 µg/day/mouse), obtained from Dr. A. F. Parlow from the National Hormone and Peptide Program (NHPP) of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK, Torrance, CA) was implanted subcutaneously before closing the wound using surgical staples. Denatured GH or PBS used as control had similar effects. Following surgery, mice received an i.p. injection of buprenorphin (0.01 mg/g body weight). After 14 days, the mice were euthanized and thymuses were obtained and placed on paper filter and were weighed and then snap-frozen for future analyses.

2.3. Thymocyte counting and Annexin V/ propidium iodide (PI) staining for apoptotic cell analysis

The thymuses of BALB/c mice of various ages were removed, single cell suspensions were prepared, and cell viability and counts were determined using a hemacytometer with 0.4%

trypan blue. Counts were also performed for each cell preparation using a Coulter cell counter (Coulter; Hialeah, FL) and mean cell counts calculated. Thymocyte cell suspensions were also used to analyze apoptosis by staining for Annexin V-FITC and the DNA dye PI (PharMingen/BD, San Jose, CA). The percentage of annexin V FITC⁺PI⁻ was verified following FACS analysis, as previously described (Vermes *et al.*, 1995).

2.4. Histological staining

Snap-frozen thymuses were embedded in OCT compound (Sakura Finetek, Torrance, CA) or fixed in 10% buffered neutral formalin solution, dehydrated and embedded in paraffin as described previously (de Mello-Coelho *et al.* 2010). Frozen sections of 5 µm were mounted on polylysine-coated slides and dried overnight at room temperature before hematoxylin and eosin (H&E) staining. Paraffin tissue sections were mounted on polylysine-coated slides, hydrated, placed at 60°C in a solution of 0.01 M sodium citrate buffer (pH 6.0) for 40 minutes and stained with Harrys hematoxylin (Lerner Laboratories, Pittsburgh, PA), rinsed and differentiated with 1% acidic alcohol before staining with eosin (Lerner Laboratories, Pittsburgh, PA). Sections were then dehydrated by transfer through a graded alcohol series, and were then mounted in organic media (CytosealTM60, Stephens Scientific, Riverdale, NJ). For lipid staining, frozen thymus sections were incubated with oil Red O (Fischer Scientific, Hanover Park, IL) diluted in 50% propylene glycol for 4 hours at room temperature and rinsed with 50% isopropyl alcohol and distilled water. Sections were then stained with hematoxylin for 2 min, rinsed with 5% ammonia, blot dried and mounted in a 50% glycerol solution.

2.5. Peroxidase immunohistochemistry

Tissue arrays were manually constructed using thymuses of mice with 2, 12 and 18–24 months of age (n=4, per age group). Thymus tissue array sections of 5 µm were deparaffinized in xylene and re-hydrated by sequential incubation in solutions with decreasing concentrations of alcohol. A standard two-step Dako Autostainer automated staining system was used (Dako Corporation, Carpinteria, CA). An antigen retrieval procedure was used to unmask antigenic sites in tissue sections. Primary purified goat anti-mouse TNF-α (R&D Systems, Minneapolis, MN) or monoclonal rat anti-mouse CD204 (2F8, Serotec, Raleigh, NC) antibodies were applied at 10 µg/ml for 60 minutes at room temperature in a humidified chamber. Unspecific IgG was used as a negative control (PharMingen/BD). After washings, tissue sections were incubated with horseradish peroxidase-conjugated donkey anti-goat or goat-anti-rat secondary antibodies (Santa Cruz Biotechnology, Dallas, TX), washed thoroughly and immersed in a freshly prepared chromogen/ substrate reagent (diaminobenzidine/hydrogen peroxide). PBS (pH 7.4) was used for all inter-step washes. Hematoxylin was used for counter-staining. Thymus sections were dehydrated in a graded alcohol series and mounted using an organic media.

2.6. TUNEL staining

A terminal deoxynucleotidyl-transferase dUTP nick end-labeling kit was used per the instructions of the manufacturer (Chemicon Int., CA). Briefly, 5 µm thick thymus sections were deparaffinized and hydrated, treated with proteinase K for 20 min at 37°C, washed in PBS for 5 min and incubated in a solution containing 3% H₂O₂ for 5 min before PBS

washing. The tissue sections were incubated with a buffer containing terminal deoxynucleotidyltransferase (TdT) enzyme in a humidified chamber at 37°C for 1 hour. After washing with PBS, tissue sections were treated with a peroxidase-conjugated anti-digoxigenin antibody for 30 min in a humidified chamber at room temperature and the staining was visualized following incubation with diaminobenzidinetetrahydrochloride and H₂O₂ enzyme substrate. Counter-staining was performed with nuclear fast red (Sigma, St Louis, MO). Apoptotic cells exhibited a brown staining in the nucleus under bright field optics.

2.7. ELISA to detect TNF- α in the supernatant of explant cultures

Fresh thymic tissue from 14–16 month-old mice that had been infused with PBS or GH for 7 days were cultured in 1 ml of RPMI 1640 + 10% FBS. After 24 hours, culture supernatants were collected and the levels of murine TNF- α were determined using a commercial ELISA kit according to manufacturer's instructions (Biosource, Camarillo, CA).

2.8. Triple quadrupole tandem mass spectrometry quantification of sphingomyelins, ceramides, gangliosides, cholesterol, and lipid peroxides

Lipid extraction and handling of sphingolipids, cholesterol, and lipid peroxides were performed using borosilicate-coated glass tubes, pipettes and injectors. Frozen tissue samples (20 – 30 mg wet weight) were suspended in a 4 ml test tube containing 10 volumes (10 μ L per 1 mg tissue) of ice cold deionized water and 30 volumes (30 μ L per 1 mg tissue) of ice cold HPLC grade methanol containing 30 mM ammonium formate, 1 mM BHT (antioxidant), and 250 nM sphingolipid internal standard mix (LM-6002; Avanti Polar Lipids Inc., Alabaster, AL) then homogenized on ice using a Pro 200 micro-homogenizer (Pro Scientific, Oxford, CT) for 1 minute at 15,000 rpm; a 3 μ l aliquot was removed to measure total proteins using Sekisui Diagnostics kit 200-55. Next, 40 volumes (40 μ l per mg tissue) of ice-cold HPLC grade chloroform were added. The mixture was vortexed 3 times and centrifuged at 1000 g for 10 minutes. The bottom (chloroform) layer was removed using a pipette and analyzed by direct injection into a tandem mass spectrometer. The top (aqueous phase) was used for analysis of d18:1 based gangliosides.

Electrospray ionization tandem mass-spectrometry (ESI/MS/MS) analyses were performed using previously published methods (Cutler *et al.* 2004; Haughey *et al.* 2004). In brief, samples were injected using a Harvard Apparatus pump at 15 μ l/min into an electrospray ionization (i.e. Turbo Ion Spray module) Sciex API 3000 triple stage quadrupole tandem mass spectrometer (Sciex Inc., Thornhill, Ontario, Canada) operating in the positive mode. The ion spray voltage (V) was 5,500 at a temperature of 80°C with a nebulizer gas of 8 psi, curtain gas of 8 psi, and the collision gas set at 4 psi. The declustering potential was 80V, the focusing potential 400V, the entrance potential –10V, the collision energy 30V, and the collision cell exit potential was 18V. The MS/MS scanned from 300 to 2,000 atomic mass units (amu) per second at a step of 0.1 amu. Each species of sphingolipid, ceramide, ganglioside, cholesterol, and lipid peroxide standard was initially identified by a Q1 mass scan, and then CAD gas breakdown products unique to each species were identified. Samples were injected into the ES/MS/MS for 3 minutes, where the mass counts accumulated and the sum of the total counts under each peak were used to quantify each

species. Positive identification and quantification of each species was achieved by precursor ion scanning (d18:1 based sphingomyelins at 184.1 amu, ceramides at 264.1 amu, dihydro SM 186.1 amu, C52 triacylglycerides at 577.9 amu, and 4-hydroxy-2-nonenal at 73.0 amu) or neutral loss scanning (cholesterols at 369.7 amu) of a purified standard. Ceramides (C16:0 – C24:1), sphingomyelins (C16:0 – C18:0) and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). Sphingomyelin C20:0 – C24:0 was purchased from Sigma Chemicals (St. Louis, MO). Palmitoyl-lactosylceramide C16:0–C16:0, stearoyl-lactosyl-ceramide C16:0–C18:0, lignoceryl-glucosyl-ceramide C16:0–C24:0, lignoceryl-galactosyl-ceramide C16:0–C24:0, and stearoyl-galactosyl-ceramide-sulfate C18:1–C24:0, and triacylglyceridepalmitic-oleic-stearic (C52, POS) were purchased from Matreya Inc. (Pleasant Gap, PA). 4-hydroxynonenol (4-HNE) was purchased from Cayman Chemicals (Ann Arbor, MI).

2.9. Statistical analyses

All data were analyzed by ANOVA, unless indicated, using GraphpadInStat software by GraphPad Software, Inc., (San Diego, CA). Values are expressed as the mean $\pm$ SEM. Differences between samples were found to be significantly different as determined by an F test ($p < 0.05$). When the differences among the individual lipid species were determined to be significant they were then analyzed by Duncan's multiple range test.

3. Results

3.1. Age-related morphological alterations and pro-inflammatory molecules expression within the thymic parenchyma

The thymus undergoes profound morphological changes during the process of aging. The young mouse thymus is characterized by very well defined cortical and medullary regions, with distinct lobules bounded by connective tissue septa that emerge from the capsulae (Figure 1). With the aging process, there is a decrease of thymocyte cellularity (Table 1). Histological analysis of thymus sections from mice at 2, 12 and 18 months of age ($n=4$, for each age group) reveal an age-related decrease of cortical thymocytes and a corresponding increase in adipose tissue, initially in the perivascular space region (Figure 1A). A progressive increase of lipid-laden unilocular and multilocular cells was observed inside the thymic parenchyma with aging (Figure 1A, B). Thymocyte apoptosis was also observed in the aging thymus, as shown by TUNEL assay analysis (Figure 1C). As expected, cells with DNA fragmentation were visualized in the cortical-medullary region of the thymus of 2 month-old mice, since T cells that do not pass through the process of thymic selection die by apoptosis. A larger distribution of apoptotic cells was visualized in the thymic parenchyma of 12 and 18 month-old mice in comparison with the youngest group of mice. Although more apoptotic cells were visualized in thymus sections of older mice, the number of TUNEL-positive cells in any one tissue section was low, presumably because of the rapid recognition and ingestion of apoptotic cells by thymic macrophages (Platt *et al.* 2000).

The expression of TNF α (Figure 1D), a pro-inflammatory cytokine involved in lymphocyte programmed cell death, and of CD204, were identified in the aging thymic parenchyma (Figure 1E). CD204 is a scavenger receptor for oxidized low density lipoproteins (LDL) that

is involved in, although not essential for, clearance of dead cells in embryogenesis (Komohara *et al.* 2005). We found that levels of TNF α and CD204 immunoreactive cells were apparently elevated in the older thymus as compared with control from young mice (Figure 1E).

Altogether, our data suggest that local basal inflammation, oxidative stress and lipodystrophy occur in the thymus during aging.

3.2. Age-related alterations in the lipid profiles of whole thymus and isolated thymocytes

Because inflammation and oxidative stress can alter lipid metabolism, and vice-versa, we performed lipidomic profiling of the thymus during aging using samples of whole thymus or pools of thymocytes isolated from mice of 4, 6, 12 and 18 months of age to analyze their lipid content by ESI/MS/MS.

Analysis of whole thymus revealed a significant accumulation of triacylglycerides (TG) ($p < 0.001$) with advancing age (Figure 2A). The amount of C52 POS (palmitic, oleic and stearic fatty acids) TG in the thymus rose sharply between 4 and 6 months of age and then continued to accumulate in the organ with aging ($p < 0.001$) (Figure 2A). On the other hand, the quantitative analysis of C52 POS TG in the pool of thymocytes isolated from young and older mice, showed a trend to decrease with age (Figure 2B). There was a significant increase in the level of free cholesterol in the whole thymus between 4 and 12 months of age ($p < 0.05$), and then a significant decrease of free cholesterol levels between 12 and 18 months of age ($p < 0.005$) (Figure 2C). In contrast, there was a significant decrease of free cholesterol levels in thymocytes isolated from 12 month-old mice compared to thymocytes isolated from 4 month-old mice ($p < 0.01$) (Figure 2D). The levels of cholesterol ester increased significantly between 4 and 18 months of age in the thymus ($p < 0.001$) (Figure 2E). Elevated levels of cholesterol ester were also observed in isolated thymocytes from mice between 4 and 12 months of age ($p < 0.05$) (Figure 2F). These results indicate that the age-related increase in the levels of TG and free cholesterol in the thymus are not the result of the accumulation of these lipids in thymocytes. Our data revealing altered cholesterol metabolism in thymocytes and, possibly, in other thymic stromal cells, which include macrophages (Youm *et al.* 2012), adipocytes or intrathymic lipid-laden cells (Langhi *et al.*, 2015), suggest that increased accumulation of lipids could contribute to maintenance of a basal inflammatory state and increased oxidative stress that adversely affect thymocytes during aging.

3.3. Lipid peroxidation increases in the whole thymus and thymocytes during aging

It is known that cellular oxidative stress can result in the peroxidation of lipids when oxygen radicals (principally hydroxyl radical and peroxynitrite) react with polyunsaturated fatty acids of membrane lipids resulting in the liberation of cleavage fragments, including the cytotoxic aldehyde 4-HNE (Uchida, 2003; Mattson, 2009). Analysis of whole thymus samples obtained from 4, 6, 12 and 18 month-old mice revealed a significant 2-fold increase of 4-HNE adducts between the ages of 6 and 12 months of age ($p < 0.05$), with their levels remaining elevated in thymuses from 18 month-old mice (Figure 2G). Thymocytes isolated from the thymuses of 12 month-old mice exhibited more than 10-fold greater amounts of 4-

HNE adducts as compared with thymocytes isolated from 4 month-old mice ($p<0.01$) (Figure 2H). These results show that thymocytes of aged mice experience a large increase in lipid peroxidation compared to thymocytes in young mice.

3.4. Age-related changes in the levels of sphingolipids and ceramides in the thymus and isolated thymocytes

Altered metabolism and accumulation of certain sphingolipids and ceramides occur in several different tissues during normal aging (Obeid and Hannun, 2003; Cutler *et al.*, 2004) and in age-related disorders that involve inflammation and oxidative stress (Cutler *et al.* 2004; Haughey *et al.* 2004; Bikman, 2012). Therefore, we measured levels of sphingomyelin and ceramide species in total thymus and isolated thymocyte samples from mice of different ages (Figure 3). Levels of sphingomyelins with carbon-chain lengths from 16 to 22 were similar in the thymuses of 4, 6 and 12 month-old mice, whereas levels of C18:1 increased in the thymuses between 4 and 6 months of age and then decreased progressively in the organ of older mice ($p<0.05$) (Figure 3A). Moreover, C24:0 and C24:1 sphingomyelins increased significantly in the thymus of mice from 4 to 12 months of age ($p<0.05$) (Figure 3A). Significant decreases in levels of C18:0, C18:1, C20:0 and C24:0 sphingomyelins occurred between 12 and 18 months of age (Figure 3A). There was a significant increase in the level of C16:0 ceramide between 4 and 12 months of age ($p<0.05$) (Figure 3B). Levels of sulfatide C24:1 and ganglioside GD1a decreased significantly between 4 and 12 months ($p<0.01$), and then were significantly elevated in 18 month-old mice ($p<0.05$) (Figure 3B). Levels of other ceramides were not significantly affected by aging. Analysis of sphingomyelin species in thymocytes isolated from 4 and 12 month-old mice, we found a significant age-related decrease of C16:0, C18:0, C18:1, C20:0, C24:0 but not of C22:0 and C24:1 sphingomyelin species (Figure 3C). In the case of ceramides, the amount of C24:1 in thymocytes isolated from 12 month-old mice was significantly less than in thymocytes from 4 month-old mice, while there were no significant age-related changes in any of the other ceramides (Figure 3D). We verified a trending increase in the amount of C16:0 and C18:0 while the level of ganglioside GD1 a in thymocytes isolated from 12 month-old mice was more than 10-fold greater than in thymocytes from 4 month-old mice (Figure 3D). Decreased levels of sphingomyelin were associated with increased ceramide levels and ganglioside GD1a levels in thymocytes during aging, which was correlated with increased thymic T cell death.

3.5. Growth hormone administration in middle aged mice increases thymocyte numbers and decreases apoptosis and ex vivo TNF- α secretion by thymic cells

It was previously proposed that growth hormone (GH) treatment can rejuvenate the thymus (French *et al.*, 2002; Napolitano *et al.*, 2008). To determine the impact of GH on the thymus, we administered subcutaneously either GH (40 $\mu\text{g}/\text{day}$) or denatured GH (dGH; 40 $\mu\text{g}/\text{day}$) as control, to middle age (12–14 month-old) mice for a period of 14 days using osmotic minipumps. The thymus of dGH-treated middle-aged mice exhibited extensive lipodystrophic features evident upon gross examination (Figure 4A), which were notably ameliorated in GH-treated mice (Figure 4B). Examination of H&E-stained sections of thymuses suggested that GH promoted an increase in thymocyte number in the thymic cortical region compared with thymus sections of control mice (Figure 4C–D). This effect

was confirmed when we quantified total thymocytes of mice treated with GH compared with control mice (Table 1). Moreover, the increase in percentage of annexin⁺ thymic apoptotic cells observed from 3 to 18 months of age in control mice ($p<0.05$) (Figure 4E) was reduced in GH-treated mice ($p<0.05$) (Figure 4E). In addition, TNF- α levels in the culture medium of thymic explants from old mice significantly decreased after treatment with GH ($p<0.05$) (Figure 4H).

This anti-inflammatory effect of GH may favor thymic T cell proliferation and/or survival. If and to what extent the beneficial effects of GH on the aging thymus *in vivo* are mediated by direct effects on thymic cells versus indirect effects remains to be established. However, it is known that thymocytes and thymic stromal cells have receptors for GH and IGF-1 and that GH stimulates IGF-1 secretion and thymopoiesis (de Mello-Coelho *et al.* 1998; French *et al.*, 2002; Taub *et al.* 2010).

3.6. *In vivo* GH administration partially reverses age-associated lipid accumulation and oxidative stress

Evaluating the effects of GH on the lipid profile of the thymus of middle-aged mice, we found a trend towards a decreased amount of TG and a highly significant decrease in the amount of free cholesterol compared with control mice treated with dGH ($p<0.05$) (Figure 5A,B). Although GH treatment for 14 days did not significantly alter the levels of cholesterol ester in the thymus of middle aged mice (Figure 5C), it significantly reduced the amounts of 4-HNE adducts (lipid peroxidation products) in the thymus compared with mice treated with dGH ($p<0.05$) (Figure 5D). There were no significant effects of GH treatment on the levels of measured sphingomyelins (Figure 5E). GH treatment did not affect the levels of any of the ceramides measured, except for C22:0 ceramide, which was present in approximately 2-fold greater amounts in the thymuses of GH-treated mice compared to control groups ($p<0.05$) (Figure 5F). Interestingly, GH treatment resulted in significant increases in the amounts of C24:0 and C24:1 dihydroceramides (precursors of ceramide) (Geeraert *et al.* 1997) ($p<0.05$) (Figure 5G). Our results indicate that GH modulates the lipid metabolism profile and reduces oxidative stress in the thymus of middle-age mice.

4. Discussion

Our findings demonstrate major alterations in the lipid profile of the thymus, associated with oxidative stress and basal inflammation during aging. We further show that GH treatment can suppress oxidative stress and inflammation, reversing relevant age-related changes in lipid metabolism as well as regulating the expression of pro-inflammatory factors in the thymus. In agreement with a model in which changes in the lipid environment change thymus cellularity, we found that GH treatment can reverse these effects and increase the number of thymocytes in middle-age mice. These findings advance an understanding of the relationships between lipid metabolism, inflammation and oxidative stress in the process of aging of the thymus. Moreover, our findings suggest potential therapeutic approaches for preserving or enhancing thymus-related immune functions during aging.

There is a progressive increase of cells containing lipid droplets in the aging thymus, which is associated with increased apoptosis of thymocytes (Sempowsky *et al.* 2000; Langhi *et al.*

2015). Our data support previous studies showing increased T cell death in the thymus with age progress (Aspinall *et al.*, 1997), which can explain the age-associated loss of clear cortico-medullary region demarcation in the thymic lobules (Aw *et al.*, 2008) while apoptotic thymocytes are cleared by macrophages (Platt *et al.*, 2007). In the current study, we also show that the expression of CD204, a scavenger receptor for ox-LDL, increases in the thymus with age. CD204 is involved in processes of cell death (Todt *et al.* 2008). Moreover, we found that TNF- α , a factor related to processes of inflammation and death, is expressed in the aged thymus. Other pro-inflammatory cytokines are expressed in the old thymus, including IL-6, Oncostatin-M and LIF. The expression of these cytokines increases with age and promote atrophy of the mouse thymus following *in vivo* injection (Sempowsky *et al.*, 2000). Intrathymic adipocytes and lipid-laden cells, which increase with aging, express LIF (Sempowsky *et al.*, 2000; de Mello Coelho *et al.*, 2004; Langhi *et al.* 2015). Adipose tissue cells also can secrete pro-inflammatory factors, such as IL-1, IL-6 and TNF- α (Rajala and Scherer, 2003).

We hypothesize that the age-related decrease in GH (Rudman *et al.*, 1990), an activator of lipolysis, leads to increased lipid accumulation in the thymic stroma and changes the environmental milieu that initiates a self-propagating cycle of adipocyte differentiation, inflammation and oxidative stress. During aging, changes in hormones, lipid metabolism, inflammation and oxidative stress, are highly interactive. Previous reports have shown that adipocytes from old animals secrete more TNF α compared to young adipocytes (Kirkland *et al.* 2002; Bikman, 2012).

Basal inflammation and the presence of lipid-laden cells and thymocyte death in the aging thymus led us to analyze the lipid profile as well as the oxidative stress status of the organ to better understand how these parameters are correlated during the process of age-associated thymic involution. We found an increase of TG levels, lipid droplets and adipose cells in the thymus with advancing age. The latter findings are consistent with previous reports that unilocular and multilocular lipid-laden cells progressively infiltrate the thymus during aging (Steinman *et al.* 1985; de Mello-Coelho *et al.* 2010; Langhi *et al.* 2015). Our analysis of lymphocytes isolated from young and middle-age mice showed a trend to decrease their TG levels with age, therefore establishing that the accumulation of TG in the thymus may occur in other cell types, including adipocytes. The lower levels of TG in thymocytes from middle-aged compared to young mice suggest the possibility of differences in lipid metabolism among thymocyte subsets because the percentage of CD4⁺CD8⁺ cells in the thymus decreases during aging.

Our measurements of levels of free cholesterol in the total thymus revealed an overall increase in cholesterol levels between 4 and 12 months of age and then a decrease between 12 and 18 months of age. These changes in cholesterol levels could be due to adipocyte infiltration that increases with aging, accompanied by alterations in cholesterol metabolism (Chui *et al.* 2011). In this regard, we showed a significant increase of cholesterol esters in the thymus with aging and it is known that adipocytes as well as foam cells can accumulate cholesterol esters, which could explain the decrease of free cholesterol levels between middle and old age in the thymus. Youm *et al.* (2012) demonstrated an increase of free cholesterol in the mouse thymus between 1 and 12 months of age as well as

immunoreactivity for filipin, which binds to unesterified cholesterol, in CD11b⁺ macrophages in thymus sections from mice with 24 months of age. On the other hand, they did not find differences in the levels of cholesterol ester between young and middle-aged thymus (Youm *et al.* 2012). Our findings of a trending decrease of TG in aged thymocytes relative to the whole tissue supports previously published results of reduced TG pathway enzymes (e.g. glycerol-3-phosphate acyltransferase-1) with age and T cell number (Collison *et al.* 2008; Gulvady *et al.* 2013). However, our data on the significant decrease of free cholesterol and increased accumulation of cholesterol esters in these cells adds to the knowledge base of this field of study.

The lipid peroxidation product 4-HNE covalently modifies proteins on lysine, histidine and cysteine residues and is a reliable marker of cumulative oxidative stress (Mattson, 2009). Moreover, 4-HNE can impair the function of a range of cellular proteins that are critical for the function and survival of cells; for example, 4-HNE binds to the GTP-binding protein Gq11 where it impairs signaling of ligands that couple to Gq11, and 4-HNE impairs the function of ion-motive ATPases and glucose transporters (Mattson, 2009). Although the potential effects of 4-HNE on thymocyte function and survival have not been established, it has been shown that 4-HNE can trigger apoptosis of cultured Jurkat T cells (Larini *et al.* 2004). Therefore, 4-HNE at the levels we measured, may contribute to the dysfunction and death of thymocytes during aging. Levels of 4-HNE were reduced in the thymuses of GH-treated mice compared to control mice, suggesting that suppression of lipid peroxidation is involved in the mechanism by which GH reduces local inflammation in the thymus and preserves thymocytes (Figure 6).

Our data indicate that, during aging, the levels of sphingomyelins decrease while the levels of ceramides increase in the thymus. These findings suggest enhanced generation of ceramides from their precursor sphingomyelins during aging. In the thymus and isolated thymocytes we found an increase and a trending increase of C16:0 ceramides, respectively. In another system, Jurkat T cells submitted to ionizing radiation for 24 hours increased C16:0 ceramide levels during apoptosis, affecting mitochondrial activity and ROS production. The authors also showed TNF- α as another inducer of C16:0 ceramide increase in Jurkat T cells (Hannun, 1994). In this sense, accumulation of ceramides in thymocytes could further promote apoptosis. Youm *et al.* (2012) also found an increase in ceramide levels in the middle-age thymus in relation to the young control. In addition, ceramides were identified in medullary macrophages in the old thymus as activation of Nlrp3 inflammasome.

Previous studies have shown similar increases of long-chain ceramides in the brain during aging (Cutler *et al.* 2004). Ceramide accumulation is commonly associated with inflammation and oxidative stress (Pettus *et al.* 2004), and it is implicated in the cellular dysfunction and degeneration that occur in a range of age-related diseases including metabolic syndrome, diabetes (Summers, 2006) and Alzheimer's disease (Cutler *et al.* 2004). Ceramide generation may mediate the apoptosis of thymocytes induced by glucocorticoids and ATP (Marchetti *et al.* 2003; Lepine *et al.*, 2006). Ganglioside GD1a was also increased in the thymus and in isolated thymocytes with age. Our findings of elevated levels of ceramides, GD1a and TNF- α pro-inflammatory cytokine associated with increased

apoptosis of thymocytes during aging are therefore consistent with a role for ceramides in age-related thymic involution (Figure 6).

GH reversed several biochemical alterations that occur in thymus during aging. Within 14 days of hormone treatment, the levels of the pro-inflammatory cytokine TNF- α and the lipid peroxidation product 4-HNE in the thymus were reduced. We did not observe any major effects of GH treatment on levels of sphingomyelins and ceramides or ganglioside GD1a in the thymus. Interestingly, however, levels of dihydroceramides were significantly elevated in the thymuses of mice treated with GH. Previous studies have shown that dihydroceramide and inhibition of dihydroceramide desaturase can protect cells against apoptosis (Breen *et al.* 2013; Siddique *et al.* 2013). Our results show that GH treatment increases thymocyte cellularity, and suggest that the mechanism involves decreasing lipid accumulation and lipotoxicity. Growth hormone treatment could also be locally regulating other known pathways involved in promoting thymocyte growth and survival. Thymocytes express GH and IGF-1 receptor on cell membrane (de Mello-Coelho *et al.* 1998; French *et al.* 2002; Taub *et al.* 2010), and it is therefore possible that GH is acting directly on thymocytes. GH also could be modulating several other factors that could contribute to promote thymocyte survival. Thymic epithelial cells have receptors for GH and IGF-1 and could be stimulated by GH to produce cytokines, thymulin and paracrine factors that can influence thymocyte physiology. However, given the wide range of GH-responsive cell types in tissues throughout the body, it is reasonable to consider intermediary roles for signals emanating from such tissues in the regenerative effects of GH on the thymus.

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Highlights

- Basal inflammation, oxidative stress and lipotoxicity occur in the aging thymus
- Lipid peroxidation increases in total thymus and thymocytes with age
- Aging decreases sphingomyelin levels and increases ceramides content in thymocytes
- Short treatment with GH reduces levels of free-cholesterol and 4-HNE in aged mice
- GH increases dihydroceramides locally and decreases thymocyte apoptosis in aged mice.

Figure 1. Changes in the morphology and expression of related pro-inflammatory molecules within the thymus during aging

(A) Representative photomicrographs of sections of thymus from young, middle-age and old mice stained with hematoxylin and eosin. In thymus sections of 2 month-old mice, the delimitation between cortical and medullary regions is well defined. In the thymuses of 12 and 18 month-old mice, adipocytes (a) are seen in the thymic septa (s) and in the subcapsular region (ca). Asterisk indicates a region of disorganization of the thymic parenchyma and decreased number of thymocytes. **(B)** Frozen sections of thymuses from 2, 12 and 24 month old mice stained with the lipid marker Oil Red O (red) and counterstained with hematoxylin dye (blue) reveals lipid droplet-containing multilocular cells (arrow heads) (n=4 for each age group). **(C)** Thymus sections stained using the TUNEL assay and counterstained with hematoxylin. Apoptotic cells (arrowheads) were stained in brown in the cortical/medullary region in the younger thymuses and in distinct regions of the parenchyma in older thymuses. **(D, E)** Immunohistochemical staining of thymus sections of mice with 2, 12 and 18 months of age using antibodies against TNF- α or CD204. Arrows indicate positive staining for these molecules in the thymic parenchyma. Counterstaining was done with hematoxylin. a, adipocyte; c, cortex; ca, capsule; md, medulla; s, septa; m, months of age. In A, C, D and E paraffin blocks constructed tissue array with four thymuses for each age group was used. Original magnification: (A, B) 400 $\times$; (C) 630 $\times$; (D, E) 1000 $\times$.

Figure 2. Age-related changes in the levels of triacylglycerides, free cholesterol, cholesterol ester and the lipid peroxidation products 4-HNE in the thymus or isolated thymocytes
ESI/MS/MS analyses were performed for C52 POS triacylglyceride levels as well as for levels of free-cholesterol, cholesterol ester and 4-HNE adducts in samples of whole thymus (A,C,E,G) or isolated thymocytes (B,D,F,H). For A, C, E and G values (cps/ mg of protein) are the mean and SE (n=4, for total thymus group). For B, D, F and H values (cps/ mg of protein/ 10⁶cells) are the mean and SE calculated from three separate samples of thymocytes obtained from thymuses of mice. Age groups are indicated in the X axes of the graphs. m, months of age. *p < 0.05; **p < 0.01, and ***p < 0.001.

Figure 3. Age-related alterations in the levels of sphingomyelin and ceramides in thymus and isolated thymocytes

Levels of the indicated sphingomyelins and ceramides in samples of whole thymus (**A,B**) or isolated thymocytes (**C,D**) from mice of the indicated ages. Values (cps/ mg of protein) are the mean and SE of determination made in separate samples of thymus (n=4) or isolated thymocytes (n=4). m, months of age. *p<0.05; **p<0.01 and ***p<0.001 compared to the corresponding value for 4 month-old mice.

Figure 4. Growth hormone treatment in middle-age mice reverses thymic morphological alterations and TNF α expression *ex vivo*

Representative micrographs of thymuses of 12–14 month-old mice treated with denatured GH (A) or GH (B) for 14 days using ALZET osmotic minipumps implanted subcutaneously in their back. Black and red asterisks indicate thymic lymphoid areas and heart location, respectively. Middle panels show representative frozen thymus sections of 14 month-old mice, treated with dGH (C) or GH (D) stained with hematoxylin and eosin. Ca, capsulae; s, septum; c, cortex; m, medulla. Original magnification: 200 $\times$. (E) Percentage of annexin⁺ thymocytes isolated from 3 or 18 month-old mice, treated (black bar) or not (white bar) with GH, analyzed by flow cytometry. Data are shown as mean percentage $\pm$ SD (n=4, for group). Unpaired *t* test statistical analyses indicate **p* < 0.05. (F) TNF- α secretion in the supernatant of 24 h-culture of thymuses explants of mice treated (black bar) or not (white bar) with GH was analyzed using Bio-Plex mouse cytokine panel. Data are shown as mean (pg/ml) $\pm$ SEM (n= 4, for group). Unpaired *t* test statistical analyses indicate *** *p* < 0.01. Ages and treatments are indicated in the figure. m, months of age.

Figure 5. *In vivo* GH effects on the lipid levels in the aging thymus

Levels of (A) triglycerides, (B) free-cholesterol, (C) cholesterol ester, (D) lipid peroxidation products, (E) sphingomyelin, (F) ceramide and (G) dihydroceramides (DHC). Values (cps/ mg of proteins) are the Mean and SE of determinations made in three separate samples of whole thymus (n=7 for all groups). Mice were implanted s.c. with osmotic minipumps containing GH for 14 days. * $p < 0.05$, ** $p < 0.01$.

Figure 6. Working model of the roles of oxidative stress, inflammation and alterations of lipid metabolism in age-related thymocyte death and the ameliorative effects of GH treatment

Top panel: During aging, there is an increase in the levels of pro-inflammatory cytokines, such as TNF- α , associated with increased levels of triacylglycerides in the thymus. In hypothesis, this inflammatory profile could explain the decreased levels of sphingomyelin and the increased levels of ceramide in thymocytes, probably due to an increased activity of sphingomyelinase. In addition, this scenario could explain the increase of ROS and lipid peroxidation products, including 4-HNE in thymocytes. Consequently, oxidative stress and ceramides could stimulate the release of pro-apoptotic factors by the mitochondria, which could contribute to stimulate thymocyte apoptosis. **Bottom panel:** The treatment of aged individuals with GH promotes an increased thymopoiesis. This effect could be explained at least by the influence of GH to reduce pro-inflammatory factors, ROS generation and consequently lipid peroxidation. Furthermore, GH promotes an increase in the levels of dihydroceramide, a precursor for the formation of ceramides which does not cause the release of pro-apoptotic factors. Altogether, these GH effects favor thymocyte survival.

Table 1

Age changes in thymocyte cellularity.

Age (months)	Thymocytes ($\times 10^7$) [*]	
03	13.60 $\pm$ 0.96	
12	0.72 ^{**}	4.00 $\pm$
16	0.48 ^{***}	3.00 $\pm$
22	0.32 ^{****}	0.92 $\pm$
Thymocytes ($\times 10^7$) [*]	Control	GH Thymocytes ($\times 10^7$) [*]
16	2.52 $\pm$ 0.8	4.96 $\pm$ 1.6 ^{**}

* Values are represented as average number $\pm$ standard deviation. A total of six Balb/c mice of each age group was used for thymocyte counting. ANOVA analyses show

**
p < 0.05;

p < 0.001; and

p < 0.0001. *In vivo* treatment with GH was done for 14 days in four mice with 16 months of age. PBS or denatured GH was used as control.