Original Paper

NeuroImmunoModulation

Neuroimmunomodulation 2012;19:137–147
DOI: 10.1159/000328844

Received: January 19, 2011
Accepted after revision: April 13, 2011
Published online: January 18, 2012

Expression of the Growth Hormone/Insulin-Like Growth Factor Axis during Balb/c Thymus Ontogeny and Effects of Growth Hormone upon ex vivo T Cell Differentiation

Hamid Kermani[a] Lindsay Goffinet[a] Marie Mottet[a] Gwenaelle Bodart[a] Gabriel Morrhaye[a] Olivier Dardenne[a] Chantal Renard[a] Lut Overbergh[c] Frédéric Baron[b] Yves Beguin[b] Vincent Geenen[a] Henri J. Martens[a]

[a]Center of Immunology, Institute of Pathology, and [b]Department of Hematology, University of Liège, CHU-B23, Liège-Sart Tilman, and [c]Laboratory of Experimental Medicine and Endocrinology, Catholic University of Leuven, Leuven, Belgium

Key Words
Thymus · Growth hormone receptor antagonist · Insulin-like growth factors · Fetal thymic organ cultures

Abstract
Aims: We address the question of the expression and the role of the growth hormone/insulin-like growth factor (GH/IGF) axis in the thymus. ***Methods:*** Using RT-qPCR, the expression profile of various components of the somatotrope GH/IGF axis was measured in different thymic cell types and during thymus embryogenesis in Balb/c mice. The effect of GH on T cell differentiation was explored via thymic organotypic culture. ***Results:*** Transcription of *Gh*, *Igf1*, *Igf2* and their related receptors predominantly occurred in thymic epithelial cells (TEC), while a low level of *Gh* and *Igf1r* transcription was also evidenced in thymic T cells (thymocytes). *Gh*, *Ghr*, *Ins2*, *Igf1*, *Igf2*, and *Igfr1* displayed distinct expression profiles depending on the developmental stage. The protein concentrations of IGF-1 and IGF-2 were in accordance with the profile of their gene expression. In fetal thymus organ cultures (FTOC) derived from Balb/c mice, treatment with exogenous GH resulted in a significant increase of double negative CD4–CD8– T cells and CD4+ T cells, together with a decrease in double positive CD4+CD8+ T cells. These changes were inhibited by concomitant treatment with GH and the GH receptor (GHR) antagonist pegvisomant. However, GH treatment also induced a significant decrease in FTOC *Gh*, *Ghr* and *Igf1* expression. ***Conclusion:*** These data show that the thymotropic properties of the somatotrope GH/IGF-1 axis involve an interaction between exogenous GH and GHR expressed by TEC. Since thymic IGF-1 is not increased by GH treatment, the effects of GH upon T cell differentiation could implicate a different local growth factor or cytokine.

Introduction

Growth hormone (GH) is a peptide hormone that is mostly synthesized in the anterior pituitary by somatotrope cells. GH exerts its effects through the GH receptor (GHR), which is itself a monomeric transmembrane mol-

H.K. and L.G. contributed equally to this work.

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1021–7401/12/0193–0137$38.00/0

Accessible online at:
www.karger.com/nim

Henri J. Martens, PhD
Center of Immunology, Institute of Pathology
University of Liège, CHU-B23
BE–4000 Liège-Sart Tilman (Belgium)
Tel. +32 4 366 2551, E-Mail hmartens@ulg.ac.be

ecule of the class I cytokine receptor family. GH binding causes receptor dimerization and leads to a structural modification that creates sites for kinase binding, which in turn activates JAK2 and STAT5 proteins [1]. Although the anterior pituitary is the main source of circulating GH, other GH-expressing sites have been identified, like the mammary glands [2], leukocytes and thymic epithelial cells (TEC) [3]. If this contribution to circulating GH is thought to be minor, GH could nevertheless play a role therein according to an autocrine/paracrine way or by stimulating local synthesis of IGF-1.

An 80-year-old study linked hypophysectomy with thymic atrophy in rats [4]. While neither GH nor the precise role of the thymus was known at that time, this can be seen as a seminal observation for the studies about the role of GH in immunoregulation [5]. The idea that GH can have a positive effect on the immune system and especially on the thymus first derived from the observation of fast-shrinking thymus in dwarf mice. The impoverished thymic cellularity in hypophysectomized rats and in 2 different strains of dwarf mice (Ames and Snell-Bagg) could be partly or completely reversed by supplementation with GH or IGF-1 [6–11]. Moreover, the expression of GHR both on TEC and on thymic T cells (thymocytes) at different stages of differentiation [3, 12, 13] strongly suggested that GH intervenes in the process of intrathymic T cell differentiation.

The thymus provides a unique, specialized microenvironment for T cell development involving a series of complex interactions between T cell precursors and a variety of thymic stromal cell components [14]. Upon colonization of the thymus, precursors undergo phenotypic changes involving cell surface proteins, which are used to identify T cell development into a series of checkpoints. The earliest T cell precursors in the thymus express neither CD4 nor CD8 and are so-called double negative (DN). In the course of differentiation, they will express both CD markers (i.e. double positive, DP) before ending as single positive (SP) CD4 or CD8 cells [15]. Dwarf Snell-Bagg mice exhibited thymic atrophy with a decrease in the DP thymocyte population [8, 9, 16], both of which regressed after GH administration [8, 10, 17]. However, recent results support that only when Snell-Bagg mice or hypophysectomized mice come under stress, do alterations appear in the immune system which can be corrected by hormone administration under these circumstances [5, 18]. Data from Snell-Bagg mice and other genetically modified animals also suggest that GH is not an obligate immunomodulator, but rather a stress-modulating hormone, counteracting housing- or stress-induced glucocorticoid increase with deleterious effects on the thymus and on immunity [5, 19]. Recent observations emphasize that GH also acts as a positive modulator of pre-T cell trafficking, through the induction of chemokines and extracellular matrix molecules [20, 21]. Finally, it was shown that ghrelin, a major GH secretagogue, reverses thymic involution in aged mice [22].

Since the important thymotropic properties of GH have been demonstrated in animals [23], translational research has recently been initiated in humans. Both CD4 frequency and thymus volume were significantly lower in a group of HIV-infected children with GHD versus a GHD-negative group [24]. Treatment of HIV patients with high doses of GH increased thymic mass and naïve CD4+ T cell generation, while GH supplementation of adults with GH deficiency restored two parameters of thymopoiesis (intrathymic proliferation of T cell precursors and thymic naïve T cell output) [25, 26]. The question about the thymotropic effects of GH, with regard to a direct or an indirect action of GH through local synthesis of IGF-1, remains unanswered. IGF-1 is part of the IGF family, which includes IGF-2 and insulin. IGF-1 and IGF-2 are expressed in many tissues and can act both in an endocrine and autocrine/paracrine manner. Both play a role in cell proliferation, differentiation and metabolism, mainly through interaction with the IGF type 1 receptor (IGF-1R) [27, 28], and they also take part in the regulation of immune and thymus function [29–31]. In mice, two insulin genes, *ins1* and *ins2*, are expressed but *ins2* is predominantly, if not almost exclusively, transcribed in the thymus [32, 33].

In this study, transcription of *Gh*, *Ghr*, *Igf1*, *Igf2*, *Ins2*, and *Igf1r* was quantified by RT-qPCR in different cell types and during ontogeny of Balb/c thymus, from embryonic day 14 (E14) to 5 weeks of age. Then, T cell differentiation was analyzed in the model of fetal thymus organ culture (FTOC) [34] treated with GH and/or the GHR antagonist pegvisomant. The choice of this model was motivated by the observation that the complexity of lymphostromal interplays in the thymus involves ill-defined interactions and that TEC grown as monolayer cultures no longer provide specific differentiation-inducing signals for positive selection, even when subsequently reaggregated into 3-dimensional structures [35]. FTOC is a system able to support the full program of T cell development from DN to SP T cells. Through treatment with specific factors, this system allows the analysis of T cell differentiation without any interference from endocrine counterparts, as earlier demonstrated [31, 34].

Materials and Methods

Reagents and Antibodies

FTOC were cultured in the Iscove's modified Dulbecco's medium (IMDM; BE 12-915F, Cambrex) supplemented with l-glutamine (2 mM, BE 17-605E, Cambrex), HEPES (25 mM, BE 17-737E, Cambrex), penicillin and streptomycin (100 UI/ml, 100 mg/ml, 17-602E, Cambrex) and 1 mM of sodium pyruvate (ref. BE 13-115E, Cambrex), and 10% fetal calf serum (FCS; Gibco; tested for murine FTOC by Dr. M. de Smedt, Ghent University). This medium will be referred to as complete medium. Recombinant human GH and GH antagonist (pegvisomant) were generously provided by Pfizer. Phycoerythrin (PE)-labeled anti-mouse CD4 and fluorescein (FITC)-labeled anti-mouse CD8 mAbs were purchased from Becton Dickinson and Co. Immunocytometry Systems (BDIS, Mountain View, Calif., USA).

Animals and Tissue Collection

Balb/c mice were provided by the Animal Department of Liège University. They were kept under conventional conditions with free access to food and water. Female mice were 5 weeks old at the time of sacrifice for the thymic cell purifications. For the exploration of ontogenic expression and FTOC analysis, Balb/c mice were mated overnight (16 h) and fetuses were removed from the pregnant females on various days of gestation (plug date = day 0).

To investigate gene expression and protein content, thymi were excised from 14-day-old to 19-day-old Balb/c embryos (E14–E19), from the postnatal day 1 and 2 thymus (PN1 and PN2), and from the age of 1–5 weeks. Total RNA was extracted from the thymi using RNeasy Mini Kit (catalog No. 74104; Qiagen) according to the manufacturer's instructions. DNA contamination was removed by treatment of the samples with RNase-Free DNase (Roche). RNA was dosed with Nanodrop® Technologies products. The protein extraction was performed using BD Clontech Protein Extraction and Labeling kit (Becton Dickinson) according to the manufacturer's recommendation. Protein concentrations were measured using the Bradford method (Bio-Rad).

Thymocytes from Balb/c mice were isolated by mechanical disruption, erythrocyte lysis (Hybrid-Max®, Sigma-Aldrich) and filtration through a 70-μm cell strainer (BD Biosciences). RNA was extracted as described above. TEC were isolated from Balb/c thymus by successive enzymatic digestions. Thymi were cut into small pieces, suspended in 5 ml of DPBS per thymus and stirred for 30 min at 4°C. Supernatant was removed, and 3 enzymatic digestions were performed with Hanks' balanced salt solution (4 ml/thymus, 2 ml/thymus and 1 ml/thymus, respectively; Lonza, Belgium) supplemented with collagenase D (1 mg/ml; Sigma-Aldrich) and DNase I (10 μg/ml; Roche Diagnostics) at 37° C for 12, 12 and 5 min, respectively. The last supernatant was treated with trypsin (Lonza) 0.25%/0.02% EDTA for 10 min at 37° C. The reaction was stopped with IMDM (Lonza) containing 10% FCS (Invitrogen). In 2 cases out of 6, a sedimentation cycle was added. Four runs of sedimentation at 1 *g* were performed. The digestion supernatant was put on 10 ml of FCS (Invitrogen) for 20 min (step 1). The supernatant was removed and the pellet was used for the following sedimentations (step 2: 10 ml of FCS for 15 min, step 3: 10 ml of FCS for 15 min, step 4: 5 ml of FCS for 15 min). The number of cells was estimated by the Trypan blue exclusion test. RNA from TEC, using the same technique as described above, were also extracted immediately after purification and approximately 95% of cells were identified as TEC [36].

FTOC were performed as described by Plum et al. [34]. Briefly, 4–6 single thymus lobes from mice on fetal day 15 were placed on the surface of preboiled membrane filters (0.8 mm pore size; Nucleopore, Costar, Cambridge, Mass., USA), which were supported on blocks of surgical gelfoam (Upjohn, Kalamazoo, Mich., USA) in 2.5 ml of complete medium. The cultures were grown for 6 days at 5% CO_2, 37°C in the presence of GH (100 ng/ml, chosen after testing concentrations from 1 to 1,000 ng/ml), pegvisomant (1,000 ng/ml), and both (GH 100 ng/ml + pegvisomant 1,000 ng/ml) added daily in a 20-μl drop directly on the organs. GH and pegvisomant were prediluted, according the manufacturer's instructions, in phosphate buffer provided and mannitol and glycine were added; this is hereafter referred to as solvent. Control cultures were performed in complete medium with additive solvent alone. At the end of the cultures, cell suspensions were prepared and the percentage of living cells was estimated by the Trypan blue exclusion test.

The experimental procedures were carried out in accordance with the Ethical Committee on Animal Experimentation at the University of Liège.

RT-qPCR Analysis

250 ng of total RNA was reverse-transcribed in a total of 20 μl by the Transcriptor First Strand cDNA Synthesis Kit (Roche) using oligo (dT) primers. Reverse-transcription products (1:20) were used directly for quantitative PCR. Quantitative PCR was carried out on an iQ cycler instrument (Bio-Rad) and hypoxanthine-guanine phosphoribosyltransferase *(Hprt)* was used as the reference gene. Expression of this reference gene was neither altered by the experimental treatment, nor by the cell type studied.

For quantification of *Igf1*, *Igf2*, and *Ins2*, we used a master mix composed by iQ Supermix (Bio-Rad) with a specific TaqMan probe, with forward and reverse primers (table 1). One microliter of cDNA product was added as a PCR template to 24 μl of master mix. The following amplification cycle protocol was used: the first segment was composed by an activation of Taq (2 min, 50°C) followed by a denaturation step (10 min, 95°C). Then, the second segment consisted of 2 steps: a denaturation (15 s, 95°C), and a primer-annealing elongation step (1 min, 60°C) repeated for 40 cycles for *Igf1*, *Igf2, Ins2* and *Hprt*.

Gh, Ghr and Igf1r transcripts were analyzed using the mouse *Gh* (Mm01258408_g1), *Ghr* (Mm01303638_m1), *Igf1r* (Mm00802831_m1) and *Hprt* (Mm01324427_m1) (TaqMan® Gene Expression Assays, Applied Biosystems) with the TaqMan Universal PCR Master Mix No Amperase UNG 2 Pack (ref. 4364341, Applied Biosystems). For these last genes, relative mRNA levels were calculated using the ΔΔCt method [37].

A calibration curve for *Igf1*, *Igf2*, *Ins2*, and *Hprt* was generated from serial dilutions of a specific cDNA plasmid for each gene. The range of calibration curve was from 10^7 to 10 mol/μl. The results were calculated from the linear regression of the appropriate curve after real-time amplification.

Protein Extraction and Dosage

IGF-1, IGF-2 and INS concentrations were measured by ELISA using mouse IGF-1 Quantikine Immunoassay (R&D Systems), mouse IGF-2 Duoset ELISA (R&D Systems) and Ultrasensitive Mouse ELISA (Mercodia), respectively. These sets were used ac-

Table 1. Probe, forward and reverse primer sequences (5′→3′) for *Igf1*, *Igf2*, *Ins2* and *Hprt*, hybridization temperature and final concentration

Primer		Sequences	Hybridization °C	Final concentration, nM
Igf1	forward	CAGGCTATGGCTCCAGCATT	60	200
	reverse	ATAGAGCGGGCTGCTTTTG	60	200
	probe	6-FAM-AGGGCACCTCAGACAGGCATTGTGG-BHQ-1	60	200
Igf2	forward	GGGAGCTTGTTGACACGCTT	60	100
	reverse	GCACTCTTCCACGATGCCA	60	300
	probe	6-FAM-CAGGCCTTCAAGCCGTGCCAAC-BHQ-1	60	200
Ins2	forward	CCGGGAGCAGGTGACCTT	60	150
	reverse	GATCTACAATGCCACGCTTCTG	60	150
	probe	6-FAM-AGACCTTGGCACTGGAGGTGGCC-BHQ-1	60	100
Hprt	forward	TTATCAGACTGAAGAGCTACTGTAATG	60	300
	reverse	CTTCAACAATCAAGACATTCTTTCC	60	300
	probe	6-FAM-TGAGAGATCATCTCCACCAATAACTTTTATGTCCC-BHQ-1	60	100

cording to the manufacturer's instructions. Protease inhibitors (Complete Mini, Roche) were used for extraction procedures as well as for dosage.

Flow Cytometry

Cell suspensions were incubated for 20 min at 4°C with a 1/200 dilution of FITC-CD8 (553030, BD Biosciences) and a 1/400 dilution of PE-CD4 (553048, BD Biosciences) mAbs. Flow cytometry was performed using a FACS Canto (Becton Dickinson) equipped with an air-water-cooled blue argon laser (488 nm) powered at 100 mW (Spinnaker 160, Spectra Physics, Mountain View, Calif., USA) and with the CellQuest analysis software (Becton Dickinson). For each sample, forward and right light scatter and triple fluorescence were determined on 100,000 cells and stored in list-mode data files. Fluorescence signals were recorded on a 3-decade log scale. The green (FITC) and orange (PE) fluorescences were collected through a 530/30 and a 575/25 bandpass filter, respectively. An electronic compensation was used to correct spectral overlap between FITC and PE fluorescences.

Statistical Analyses

The Mann-Whitney-Wilcoxon rank sum test was used for the comparison of transcript expression between thymocytes and TEC. For the correlation between transcript expressions during ontogeny, we used the Spearman rank correlation test. Statistical analyses were performed on the Prism 4.0 program (GraphPad, San Diego, Calif., USA). While some variability in cell production across FTOC experiments could be observed, cell production from replicate samples within a given experiment was in close agreement. Variations in the end culture cell production percentages and numbers, based on slight differences in the developmental/gestational states of tissue used in separate experiments, as well as uncontrollable differences in culture conditions between experiments, have already been described [38, 39]. We used a representation of T cell subset population percentage compared to basal values in an effort to offer the best description of the effect of additives. Normality of all data distributions was tested using the Shapiro-Wilk W test for normality. We used the parametric one-way ANOVA with the Bonferroni multiple comparison test for normally distributed data.

Results

Ontogeny of Gene Expression in the Murine Thymus (fig. 1)

Gh expression increased in a regular way from E14 till birth and declined thereafter. As expected and already shown, *Igf2* was massively expressed in the thymus on E14, but this expression declined during fetal life and became extremely low on PN2. *Gh* expression was not measured after PN2. All other genes studied, except *Igf1r* and *Ins2*, were expressed at the highest level on E14, and their expression declined after birth. With regard to the profile of *Ins2* transcription, it was almost undetectable on all the days before birth, but its expression immediately increased on PN1 and remained well-detected until 5 weeks of age, although with some fluctuations.

Comparison of transcription levels showed a 10-fold higher expression of *Igf2* throughout the fetal period compared to *Igf1* (fig. 1e, c). *Igf2* expression was still slightly higher than *Igf1* on PN2 (3-fold, 0.66 vs. 0.24). At 4 weeks, the *Igf1* mean expression level was 0.098, while *Igf2* mean expression was 0.016. Thymic *Ins2* expression during gestation was much lower than *Igf* expression (fig. 1c, e, f). Even the increase of *Ins2* expression after birth was lower than the level of *Igf1* and *Igf2* expression

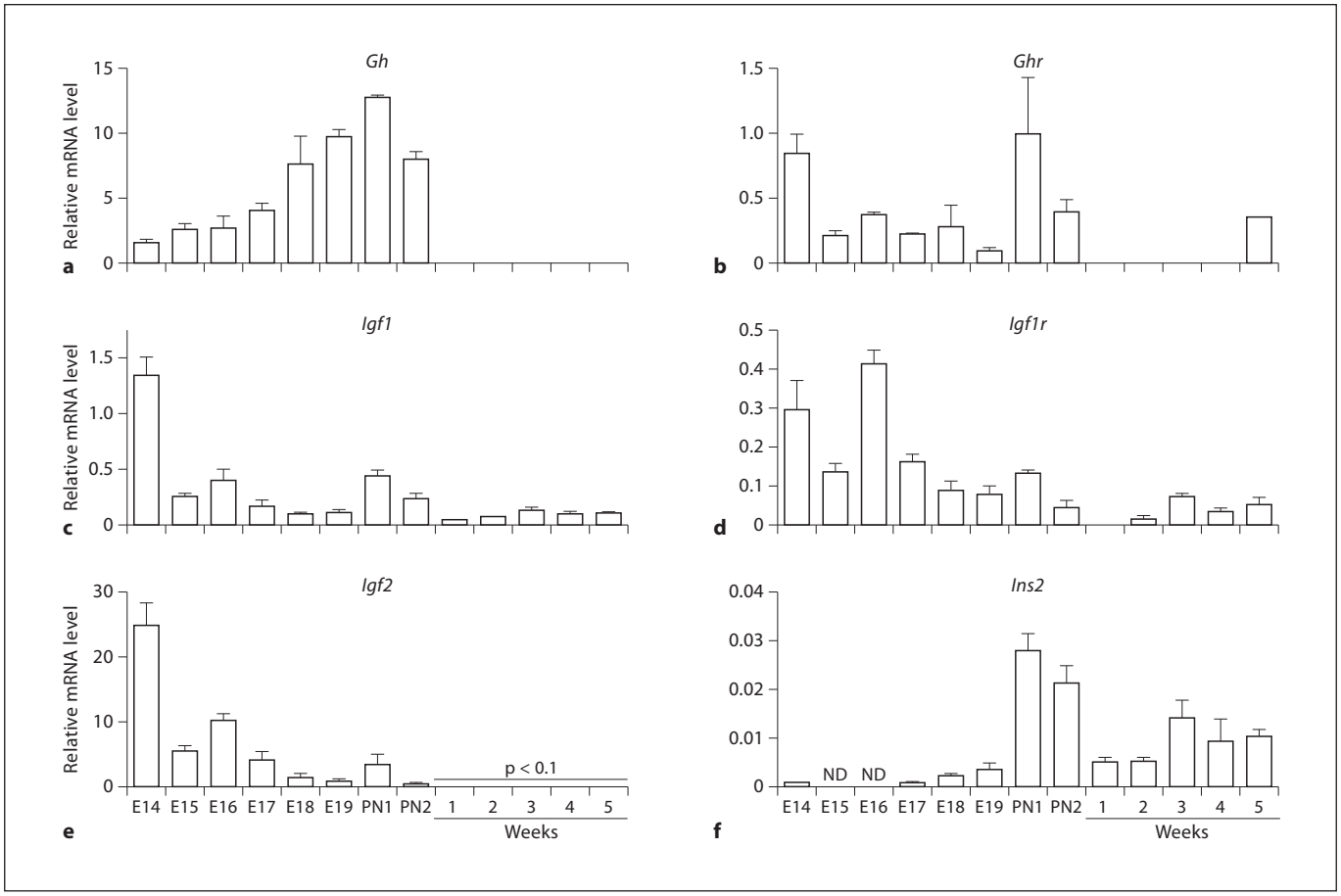

Fig. 1. Transcript level of *Gh* (**a**), *Ghr* (**b**), *Igf1* (**c**), *Igf1r* (**d**), *Igf2* (**e**) and *Ins2* (**f**) during ontogeny of the Balb/c thymus. Copy numbers of each gene transcript were normalized against *Hprt* expression and expressed as the relative mRNA level (mean ± SEM of 2–5 separate Balb/c thymus extractions). *Gh* expression was not quantified after PN2.

level in the postnatal period. The *Gh* expression level could not be directly compared due to its expression kinetics, but its overall mean level was above *Igf1*.

The profile of *Igf1* expression was very similar to the *Ghr* profile during fetal life, but not to the *Gh* profile. This was confirmed by the very positive correlation (Spearman: $r = 0.81$, $p < 0.0001$) observed between *Ghr* and *Igf1* mRNA (fig. 2a). During fetal life, a negative correlation was also observed between the levels of *Gh* and *Ghr* expression in the murine thymus (Spearman: $r = 0.75$, $p = 0.0018$; fig. 2b), but this was no longer the case after birth (data not shown).

Protein Concentrations during Ontogeny

As shown in figure 3, intrathymic IGF-1 and IGF-2 concentrations (expressed per milligram of total protein) rapidly decreased after E14. Intrathymic IGF-2 concentration declined from 4,500 pg/mg on E14 to 450 pg/mg on PN2 and 150 pg/mg at 5 weeks. Intrathymic IGF-1 concentration declined from 1,750 pg/mg on E14 to 150 pg/mg on PN2, and remained stable thereafter. Intrathymic INS concentration was very low, fluctuating between 12 and 64 pg/mg, and did not parallel the profile of *Ins2* expression (data not shown). IGF-2 predominated in the thymus during the entire fetal life, but both IGFs were found in similar concentrations after birth.

Cell Topography of Gene Expression

Cell components of 5-week-old mouse thymus were separated and mRNA expression of *Gh*, *Ghr*, *Igf1*, *Igf2*, and *Igf1r* was quantified by RT-qPCR (fig. 4). As shown, the 5 targets investigated for their gene expression levels

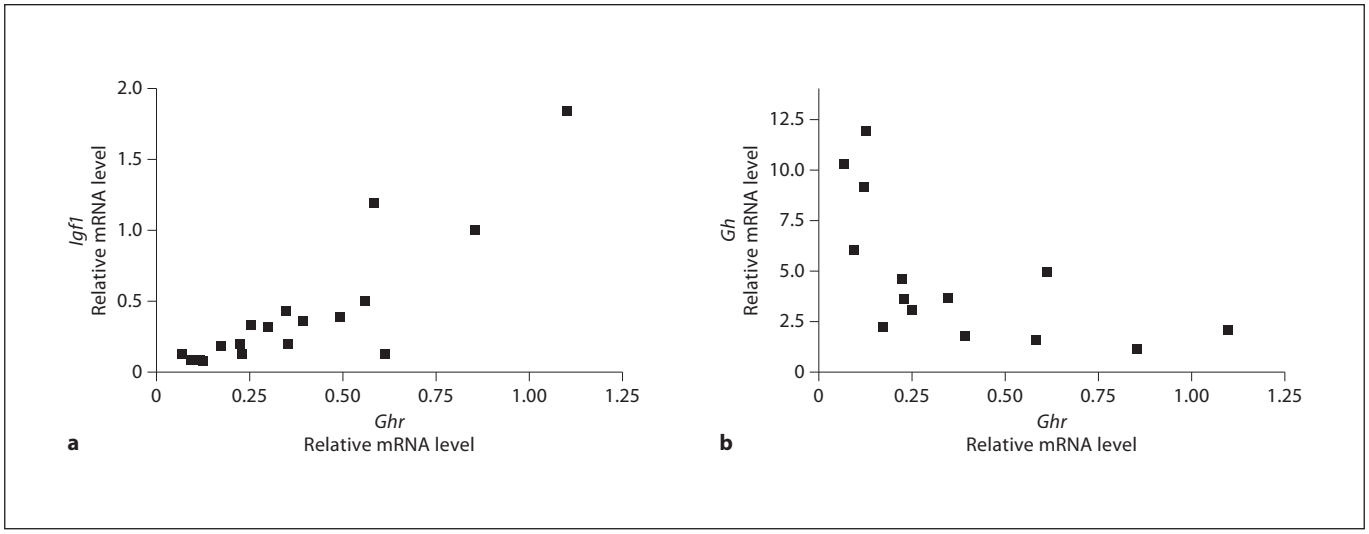

Fig. 2. Correlation of *Gh*, *Ghr* and *Igf1* expression in the fetal Balb/c thymus. Relative expression levels of *Igf1* (**a**) and *Gh* (**b**) during fetal life were plotted against *Ghr*. Correlation was calculated using the Spearman rank correlation test.

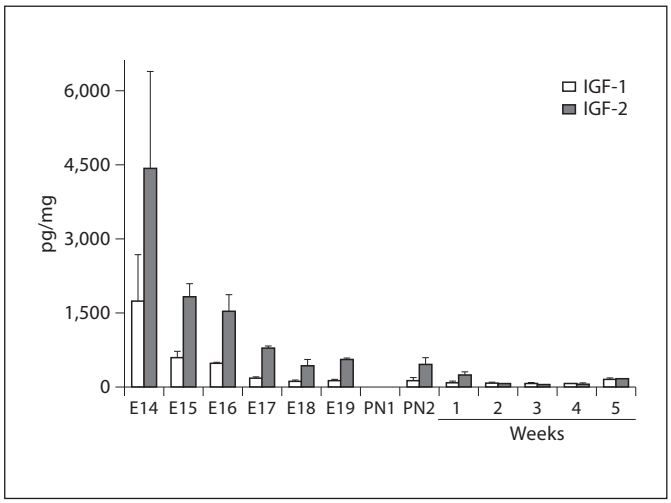

Fig. 3. Intrathymic concentrations of IGF proteins during ontogeny of Balb/c thymus. Each column represents the total amount (mean ± SEM) of IGF-1 and IGF-2 extracted from 3–5 individual Balb/c thymus.

were much lower in thymocytes than in TEC. The expression of *Hprt* used as a reference gene to normalize all measurements was similar both in TEC and thymocytes (25,042 ± 4,765 copies *vs* 25,293 ± 3,403 copies, respectively; mean ± SD, p = 0.9674). *Gh* and *Igf1r* expression was high in TEC and was also detected at a very low level in thymocytes. Expression of *Ghr*, *Igf1* and *Igf2* was virtually absent in adult purified thymocytes. At 5 weeks, thymic *Igf2* expression was lower than *Igf1*.

Effects of GH and GHR Antagonists on T Cell Differentiation in FTOC

The solvent used for predilution of GH and pegvisomant did not affect the thymocyte subpopulations in FTOC (data not shown). GH affected T cell development in FTOC established from 15-day-old fetal Balb/c mice and maintained for 6 days in culture (fig. 5). GH treatment resulted in a significant increase in the percentage of DN CD4–CD8– T cells and single positive CD4+ T cells, with a concomitant and parallel decrease in the compartment of DP CD4+CD8+ T cells. GH treatment did not affect the percentage of CD8+ T cells. Addition of the GHR antagonist pegvisomant alone did not affect T cell differentiation, but inhibited the effects of GH when used in combination.

After 6-day FTOC, thymic lobes were also lyzed and assayed for *Gh*, *Ghr* and *Igf1* expression (fig. 6). Expression of the three genes in basal conditions ranged in a similar order to the one in freshly isolated TEC with *Gh* > *Igf1* > *Ghr*. Addition of 100 ng/ml GH to FTOC decreased expression of *Gh* and *Ghr*, and was not associated with an increase of *Igf1* expression. Treatment with the GHR antagonist (pegvisomant, at 1,000 ng/ml) alone significantly decreased *Ghr* and *Igf1* expression. Treatment

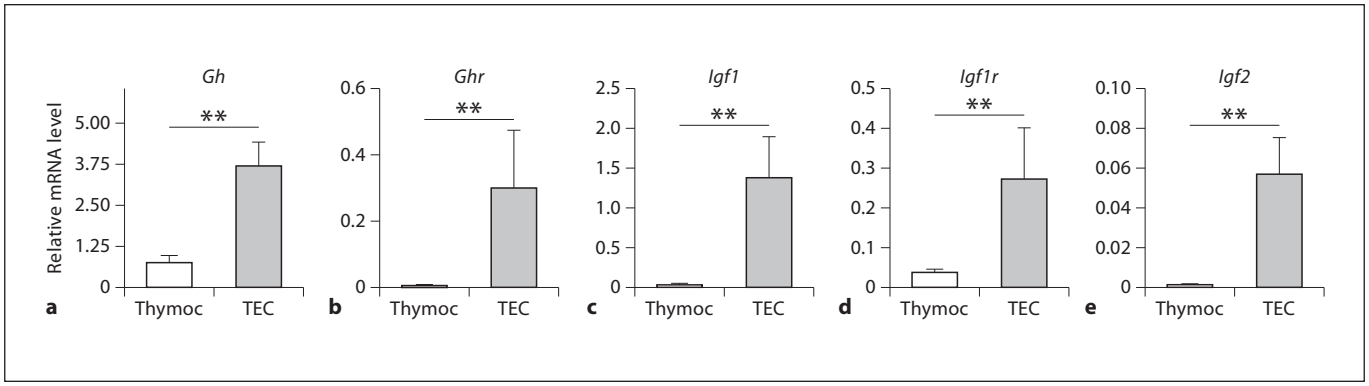

Fig. 4. Gene expression according to the thymic cell type. Transcript level of *Gh* (**a**), *Ghr* (**b**), *Igf1* (**c**), *Igf1r* (**d**) and *Igf2* (**e**) in thymocytes (n = 4) and freshly isolated TEC (n = 6) from 5-week-old Balb/c thymus. Copy numbers of each somatotrope axis member were normalized against *hprt* expression and expressed as the relative mRNA level (mean ± SEM). ** $p < 0.01$: Mann-Whitney-Wilcoxon rank sum test. Thymoc = Thymocytes.

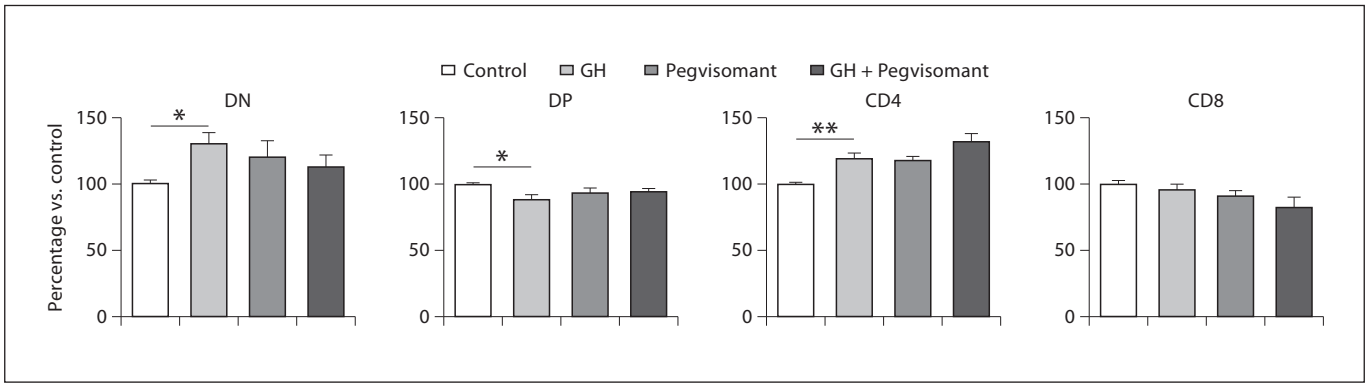

Fig. 5. Effect of GH on T cell subsets in FTOC derived from 14-day-old Balb/c. Percentage of thymocyte subsets collected from FTOC after treatment with GH 100 ng/ml and pegvisomant 1,000 ng/ml. Each column represents percentage (mean ± SEM) of thymocyte subpopulations versus the control, with solvent alone added in the total FTOC thymocytes. Data was collected from 8 experiments in triplicate. * $p < 0.05$; ** $p < 0.01$: Bonferroni multiple comparison test following one-way ANOVA test.

of FTOC with a combination of GH and GHR antagonists did not modify *Gh* and *Ghr* expression when compared to the basal condition, but *Igf1* expression was significantly lowered. When compared to the conditions of treatment with GH alone, the addition of the GHR antagonist induced a significant increase in thymic *Gh* expression.

Discussion

The first goal of this study was to investigate the intrathymic expression of GH- and IGF-related members during ontogeny of the Balb/c mouse, as well as the cell topography of this expression. Only *Gh* transcription progressively increases during fetal life. As already shown in our previous studies, *Igf2* expression is highly predominant during fetal life, and decreases from E14 to E19 to become almost undetectable after PN2. During fetal life, *Ins2* expression stands at the limit of detection but increases after birth and remains well-detected until 5 weeks of age. These observations in mice concur with a previous qualitative investigation of the human thymic IGF axis, which also detected *IGF2* and *IGF1R* transcription in cultured human TEC [40]. Along ontogenesis of *Gh*, *Ghr* and *Igf1* expression in Balb/c thymus, there is a positive relationship between the level of *Ghr* and *Igf1*

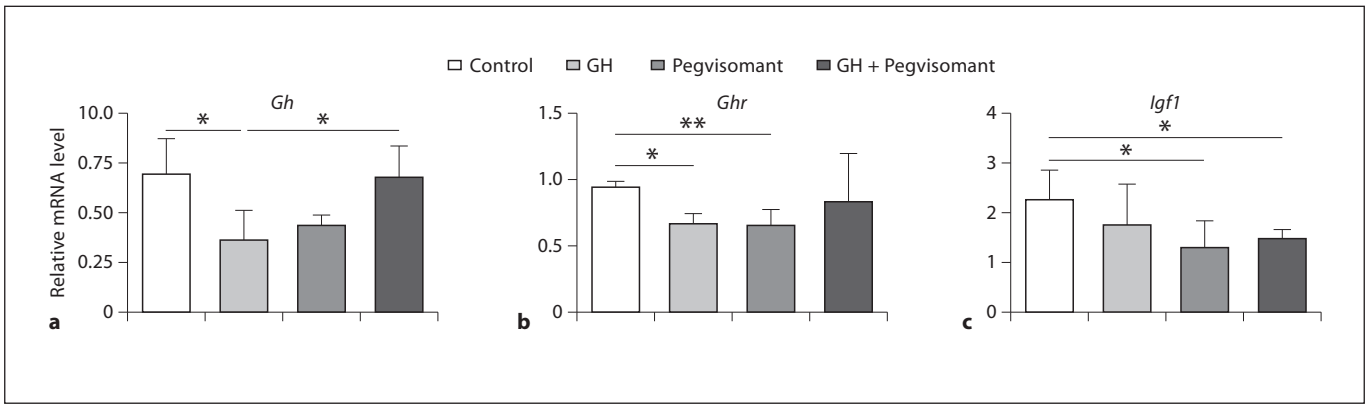

Fig. 6. Effect of GH on gene expression in FTOC derived from 14-day-old Balb/c. Transcript levels of *Gh* (**a**), *Ghr* (**b**) and *Igf1* (**c**) in FTOC after treatment with GH 100 ng/ml and pegvisomant 1,000 ng/ml. Copy numbers of each gene were normalized against *Hprt* expression and expressed as the relative mRNA level (mean ± SEM). Data collected from 4 experiments in triplicate. * $p < 0.05$; ** $p < 0.01$: Bonferroni multiple comparison test following one-way ANOVA test.

expression, and there is an inverse correlation between *Gh* and *Ghr* expression, which disappears after birth. So during fetal life, it seems that *Igf1* expression in the thymus depends rather on *Ghr* expression. These data are coherent with the GH-induced downregulation of GHR expression observed in several studies.

At the protein level, the profile of intrathymic IGF concentrations is completely superimposable to the profile of *Igf* mRNA. We were also able to detect the INS protein encoded by *Ins2* in the thymus; however, at extremely low concentrations (±1% of IGFs). After birth, IGF-2 concentration decreases to reach the same level as IGF-1. With regard to the availability of thymic insulin-related antigens that could play a role in T cell-negative selection [41, 42], the observation that *Ins2* expression appears only after birth suggests that only at that moment could INS be added to the panoply of insulin-related selecting antigens.

Only *Gh* and *Igf1r* transcription was detected in thymocytes and appeared to be in the same order of magnitude as the reference gene *hprt*. *Gh*, *Ghr*, *Igf1*, *Igf2*, *Ins2* and *Igf1r* transcription was clearly observed in murine TEC. The degree of *Gh* expression in freshly isolated TEC is 4-fold higher than in thymocytes, and this difference amends the previous data obtained with nonquantitative techniques showing that *Gh* was exclusively expressed by TEC [43]. *Ghr* expression is virtually absent in freshly isolated thymocytes. This apparent paradox with previous observations [3] can be explained when one considers that adult thymus used for isolation contains more than 80% DP thymocytes, which were GHR-negative in the cytometric analysis referred to above.

It was previously shown that GH and IGF-1 can act on hematopoietic cells including lymphocytes to mediate effects such as enhanced proliferation and/or inhibition of apoptosis [e.g. 23, 44–46]. Moreover, GH can expand the number of thymocytes in several ways. Indeed, GH can enhance TEC production of the chemokine CXCL12 (formerly known as stromal-derived factor 1 or SDF-1) [20], which is a ligand for CXCR4. CXCR4 is highly expressed on immature thymocytes [47, 48] and its activation can in turn inhibit GH function [49]. GH and IGF-1 could also stimulate the growth of TEC, which are needed to support thymic function [50]. Expanded numbers of TEC produce increased amounts of extracellular matrix material such as laminin that are essential for thymocyte adherence [51].

In rodents, IGF-1 plays an important role in pre- and postnatal growth whereas IGF-2 seems to be important essentially during fetal growth [52]. As noted, both play a role in cell proliferation, differentiation and metabolism [28] that might be mediated via IGF-1R and IGF-2R [27, 53] but also via the INS-R, although with a lower affinity. We have previously assessed the functional influence of IGF-1 and IGF-2 upon T cell differentiation in murine FTOC. Specific anti-IGF-1 antibodies decreased the DN proportion, while T cell differentiation from the DN to the DP stage was inhibited when FTOC were treated with antibodies against IGF-2, IGF-1R, and even IGF-2R. FTOC treatment with antibodies against IGF-1R or IGF-2R also severely compromised the increase in the global cellularity usually observed under control conditions. No significant effect was observed following FTOC treatment

with a specific monoclonal antibody directed to (pro)insulin [31]. Because of the low level of *Igf1r* expression by thymocytes throughout ontogenesis as demonstrated in this study, these effects could reflect an inhibition through IGF-1R expressed by TEC rather than a direct effect on thymocytes. The question remains unanswered and a recent exhaustive review has highlighted the complexity of autocrine/paracrine circuitry of GH/IGF-1 in the thymus microenvironment [23]. We therefore used the GHR antagonist pegvisomant in an effort to identify the role of thymic GH. Cytometric analyses of thymic lymphocytes after 6-day FTOC in the presence of GH indicated an increase in the proportion of DN CD4–CD8– and CD4+ T lymphocytes in parallel with a decrease in the population of DP. This may result from an increase in DN cell proliferation, compatible with the high level of GHR expression found on this subpopulation [12]. Indeed, in vivo experiments already showed that the number of primary DN thymocytes increased in old mice perfused with GH [54], while it was previously shown that GH does not affect repartition of thymocyte subpopulations in young mice [55]. However, these results must be interpreted by taking into account the GH effect on bone marrow T cell progenitors, modulation of T cell trafficking [21, 56–58] and intervention of IGF-1 as the main mediator of GH in vivo. When GH was previously tested on thymic organ cultures, it demonstrated either no effect on proliferation and T cell subset proportion [59] or a single enhanced proliferation without modification of T cell subsets [60]. The first study was performed in the presence of FCS, which contains bovine GH, while the second study was carried out in a serum-free medium. Both experiments were conducted for a very long period (19 and 13 days) compared to our 6-day FTOC. In the more recent study, the most efficient GH concentrations were 10 ng/ml and 100 ng/ml, coherent with our observations, but the DN proportion remained >40% after 13 days, while we observed only 20% after 6 days, which suggests that the serum-free medium was not able to fully support T cell differentiation. The discrepancy between these previous experiments and our own observations cannot only be attributed to the duration of FTOC but also to methods of additive delivery. While the 2 previous studies used a single addition of GH at the start of the culture, we performed daily application of a single drop directly on the organs, in an effort to mimic the pulsatile nature of in vivo GH secretion. We also observed that addition of the GHR antagonist pegvisomant had no significant effect per se but did inhibit any significant effect following GH treatment. This suggests that neither endogenous GH in TEC, nor bovine GH in FCS interfered in a significant manner under our study conditions. In addition, the inhibition of GH effects by pegvisomant strongly argues for the specificity of GH effect on the thymus.

When evaluating the levels of *Gh*, *Ghr* and *Igf1* in the organs at the end of FTOC, we found that all three relative levels had increased compared with E15 when the organ was excised. Interestingly, it seems that thymic endogenous GH regulation is partially independent of regulators of GH production in the pituitary gland, an idea already suggested more than 10 years ago [1]. A very interesting observation is the downregulation of *Ghr* expression induced by GH treatment with a parallel decrease in thymic *Igf1* transcription. These effects were partially, but not significantly, reversed by pegvisomant.

Altogether, these data argue that the thymotropic properties of the somatotrope GH/IGF-1 axis involve an interaction between GH and GHR mainly expressed by TEC, although GH treatment downregulated *Ghr* expression by TEC. A direct but very moderate effect of GH on DN CD4–CD8– T cells cannot be totally excluded. Since thymic IGF-1 is not increased by FTOC treatment with GH, the effects of GH upon T cell differentiation could involve a different local growth factor or cytokine.

Acknowledgments

These studies are supported by the National Fund of Scientific Research (NFSR, Brussels), the Fund for Research in Industry and Agronomy (FRIA, Brussels), the Wallonia Region of Belgium (DGTRE Reseaux2-SENEGENE No. 05/1/6192) and an independent research grant (Pfizer, Europe). Hamid Kermani received a grant from the Ministry of Health and Medical Education, Islamic Republic of Iran. Vincent Geenen is Research Director and Frédéric Baron is Senior Research Associate at the NFSR of Belgium.

References

1 Clark R: The somatogenic hormones and insulin-like growth factor-1: stimulators of lymphopoiesis and immune function. Endocr Rev 1997;18:157–179.

2 Mol JA, Henzen-Logmans SC, Hageman P, Misdorp W, Blankenstein MA, Rijnberk A: Expression of the gene encoding growth hormone in the human mammary gland. J Clin Endocrinol Metab 1995;80:3094–3096.

3 De Mello-Coelho V, Gagnerault MC, Souberbielle JC, Strasburger CJ, Savino W, Dardenne M, Postel-Vinay MC: Growth hormone and its receptor are expressed in human thymic cells. Endocrinology 1998;139:3837–3842.

4 Smith P: The effect of hypophysectomy upon the involution of the thymus in the rat. Anat Rec 1930;47:119–143.
5 Dorshkind K, Horseman ND: The roles of prolactin, growth hormone, insulin-like growth factor-1, and thyroid hormones in lymphocyte development and function: insights from genetic models of hormone and hormone receptor deficiency. Endocr Rev 2000;21:292–312.
6 Duquesnoy RJ: Immunodeficiency of the thymus-dependent system of the Ames dwarf mouse. J Immunol 1972;108:1578–1590.
7 Guler HP, Zapf J, Scheiwiller E, Froesch ER: Recombinant human insulin-like growth factor 1 stimulates growth and has distinct effects on organ size in hypophysectomized rats. Proc Natl Acad Sci USA 1988;85:4889–4893.
8 Van Buul-Offers S, Van den Brande JL: The growth of different organs of normal and dwarfed Snell mice, before and during growth hormone therapy. Acta Endocrinol (Copenh) 1981;96:46–58.
9 Murphy WJ, Durum SK, Anver MR, Longo DL: Immunologic and hematologic effects of neuroendocrine hormones. Studies on DW/J dwarf mice. J Immunol 1992;148:3799–3805.
10 Murphy WJ, Durum SK, Longo DL: Role of neuroendocrine hormones in murine T cell development: growth hormone exerts thymopoietic effects in vivo. J Immunol 1992; 149:3851–3857.
11 Villanua MA, Szary A, Bartke A, Esquifino AI: Changes in lymphoid organs of Ames dwarf mice after treatment with growth hormone, prolactin or ectopic pituitary transplants. J Endocrinol Invest 1992;15:587–595.
12 Gagnerault MC, Postel-Vinay MC, Dardenne M: Expression of growth hormone receptors in murine lymphoid cells analyzed by flow cytofluorometry. Endocrinology 1996;137: 1719–1726.
13 Savino W, Postel-Vinay MC, Smaniotto S, Dardenne M: The thymus gland: a target organ for growth hormone. Scand J Immunol 2002;55:442–452.
14 Anderson G, Moore NC, Owen JJ, Jenkinson EJ: Cellular interactions in thymocyte development. Annu Rev Immunol 1996;14:73–99.
15 Murphy KM, Travers P, Walport M: The development and survival of lymphocytes; in Murphy K, Travers P, Walport M (eds): Janeway's Immunobiology. New York, Garland Science, 2008, chapt. 7.
16 van Buul-Offers S, van den Brande JL: Cellular growth in several organs of normal and dwarfed Snell mice. Acta Endocrinol (Copenh) 1982;99:141–149.
17 Murphy WJ, Durum SK, Longo DL: Differential effects of growth hormone and prolactin on murine T cell development and function. J Exp Med 1993;178:231–236.
18 Foster MP, Jensen ER, Montecino-Rodriguez E, Leathers H, Horseman N, Dorshkind K: Humoral and cell-mediated immunity in mice with genetic deficiencies of prolactin, growth hormone, insulin-like growth factor-I, and thyroid hormone. Clin Immunol 2000;96:140–149.
19 Welniak LA, Sun R, Murphy WJ: The role of growth hormone in T-cell development and reconstitution. J Leukoc Biol 2002;71:381–387.
20 Smaniotto S, de Mello-Coelho V, Villa-Verde DM, Pleau JM, Postel-Vinay MC, Dardenne M, Savino W: Growth hormone modulates thymocyte development in vivo through a combined action of laminin and CXC chemokine ligand 12. Endocrinology 2005;146: 3005–3017.
21 Savino W: Neuroendocrine control of T cell development in mammals: role of growth hormone in modulating thymocyte migration. Exp Physiol 2007;92:813–817.
22 Dixit VD, Yang H, Sun Y, Weeraratna AT, Youm YH, Smith RG, Taub DD: Ghrelin promotes thymopoiesis during aging. J Clin Invest 2007;117:2778–2790.
23 Savino W, Dardenne M: Pleiotropic modulation of thymic functions by growth hormone: from physiology to therapy. Curr Opin Pharmacol 2010;10:434–442.
24 Vigano A, Saresella M, Trabattoni D, Giacomet V, di Natale B, Merlo M, Venuto A, Villa ML, Vanzulli S, Ferrante P, Clerici M: Growth hormone in T-lymphocyte thymic and postthymic development: A study in HIV-infected children. J Pediatr 2004;145:542–548.
25 Napolitano LA, Schmidt D, Gotway MB, Ameli N, Filbert EL, Ng MM, Clor JL, Epling L, Sinclair E, Baum PD, Li K, Killian ML, Bacchetti P, McCune JM: Growth hormone enhances thymic function in HIV-1-infected adults. J Clin Invest 2008;118:1085–1098.
26 Morrhaye G, Kermani H, Legros JJ, Baron F, Beguin Y, Moutschen M, Cheynier R, Martens HJ, Geenen V: Impact of growth hormone (GH) deficiency and GH replacement upon thymus function in adult patients. PLoS One 2009;4:e5668.
27 LeRoith D, Werner H, Beitner-Johnson D, Roberts CT Jr: Molecular and cellular aspects of the insulin-like growth factor 1 receptor. Endocr Rev 1995;16:143–163.
28 Jones JI, Clemmons DR: Insulin-like growth factors and their binding proteins: biological actions. Endocr Rev 1995;16:3–34.
29 Kelley KW, Meier WA, Minshall C, Schacher DH, Liu Q, VanHoy R, Burgess W, Dantzer R: Insulin growth factor-I inhibits apoptosis in hematopoietic progenitor cells. Implications in thymic aging. Ann NY Acad Sci 1998;840:518–524.
30 Beschorner WE, Divic J, Pulido H, Yao X, Kenworthy P, Bruce G: Enhancement of thymic recovery after cyclosporine by recombinant human growth hormone and insulin-like growth factor I. Transplantation 1991; 52:879–884.
31 Kecha O, Brilot F, Martens H, Franchimont N, Renard C, Greimers R, Defresne MP, Winkler R, Geenen V: Involvement of insulin-like growth factors in early T cell development: a study using fetal thymic organ cultures. Endocrinology 2000;141:1209–1217.
32 Chentoufi AA, Polychronakos C: Insulin expression levels in the thymus modulate insulin-specific autoreactive T-cell tolerance: the mechanism by which the IDDM2 locus may predispose to diabetes. Diabetes 2002;51: 1383–1390.
33 Palumbo MO, Levi D, Chentoufi AA, Polychronakos C: Isolation and characterization of proinsulin-producing medullary thymic epithelial cell clones. Diabetes 2006;55: 2595–2601.
34 Plum J, De Smedt M, Leclercq G, Vandekerckhove B: Influence of TGF-beta on murine thymocyte development in fetal thymus organ culture. J Immunol 1995;154:5789–5798.
35 Anderson G, Hare KJ, Platt N, Jenkinson EJ: Discrimination between maintenance- and differentiation-inducing signals during initial and intermediate stages of positive selection. Eur J Immunol 1997;27:1838–1842.
36 Brilot F, Chehadeh W, Charlet-Renard C, Martens H, Geenen V, Hober D: Persistent infection of human thymic epithelial cells by coxsackievirus B4. J Virol 2002;76:5260–5265.
37 Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001;25:402–408.
38 DeLuca D, Bluestone JA, Shultz LD, Sharrow SO, Tatsumi Y: Programmed differentiation of murine thymocytes during fetal thymus organ culture. J Immunol Methods 1995;178: 13–29.
39 Middlebrook AJ, Lebsack T, DeLuca D: TNF-alpha mediated modulation of T cell development and exacerbation of in vitro T1DM in fetal thymus organ culture. J Autoimmun 2007;29:134–145.
40 Kecha O, Martens H, Franchimont N, Achour I, Hazee-Hagelstein MT, Charlet-Renard C, Geenen V, Winkler R: Characterization of the insulin-like growth factor axis in the human thymus. J Neuroendocrinol 1999;11:435–440.
41 Nakayama M, Abiru N, Moriyama H, Babaya N, Liu E, Miao D, Yu L, Wegmann DR, Hutton JC, Elliott JF, Eisenbarth GS: Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice. Nature 2005; 435:220–223.
42 Geenen V, Kecha O, Brilot F, Charlet-Renard C, Martens H: The thymic repertoire of neuroendocrine-related self antigens: biological role in T-cell selection and pharmacological implications. Neuroimmunomodulation 1999;6:115–125.

43 Maggiano N, Piantelli M, Ricci R, Larocca LM, Capelli A, Ranelletti FO: Detection of growth hormone-producing cells in human thymus by immunohistochemistry and non-radioactive in situ hybridization. J Histochem Cytochem 1994;42:1349–1354.
44 Dobashi H, Sato M, Tanaka T, Tokuda M, Ishida T: Growth hormone restores glucocorticoid-induced T cell suppression. FASEB J 2001;15:1861–1863.
45 Mitsunaka H, Dobashi H, Sato M, Tanaka T, Kitanaka A, Yamaoka G, Tokuda M, Matoba K, Hiraishi T, Ishida T: Growth hormone prevents Fas-induced apoptosis in lymphocytes through modulation of Bcl-2 and caspase-3. Neuroimmunomodulation 2001;9:256–262.
46 Lempereur L, Brambilla D, Scoto GM, D'Alcamo M, Goffin V, Crosta L, Palmucci T, Rampello L, Bernardini R, Cantarella G: Growth hormone protects human lymphocytes from irradiation-induced cell death. Br J Pharmacol 2003;138:1411–1416.
47 Aiuti A, Tavian M, Cipponi A, Ficara F, Zappone E, Hoxie J, Peault B, Bordignon C: Expression of CXCR4, the receptor for stromal cell-derived factor-1 on fetal and adult human lympho-hematopoietic progenitors. Eur J Immunol 1999;29:1823–1831.
48 Zaitseva MB, Lee S, Rabin RL, Tiffany HL, Farber JM, Peden KW, Murphy PM, Golding H: CXCR4 and CCR5 on human thymocytes: biological function and role in HIV-1 infection. J Immunol 1998;161:3103–3113.
49 Garzon R, Soriano SF, Rodriguez-Frade JM, Gomez L, Martin de Ana A, Sanchez-Gomez M, Martinez AC, Mellado M: CXCR4-mediated suppressor of cytokine signaling up-regulation inactivates growth hormone function. J Biol Chem 2004;279:44460–44466.
50 Timsit J, Savino W, Safieh B, Chanson P, Gagnerault MC, Bach JF, Dardenne M: Growth hormone and insulin-like growth factor-1 stimulate hormonal function and proliferation of thymic epithelial cells. J Clin Endocrinol Metab 1992;75:183–188.
51 de Mello Coelho V, Villa-Verde DM, Farias-de-Oliveira DA, de Brito JM, Dardenne M, Savino W: Functional insulin-like growth factor-1/insulin-like growth factor-1 receptor-mediated circuit in human and murine thymic epithelial cells. Neuroendocrinology 2002;75:139–150.
52 Baker J, Liu JP, Robertson EJ, Efstratiadis A: Role of insulin-like growth factors in embryonic and postnatal growth. Cell 1993;75:73–82.
53 Morgan DO, Edman JC, Standring DN, Fried VA, Smith MC, Roth RA, Rutter WJ: Insulin-like growth factor 2 receptor as a multifunctional binding protein. Nature 1987;329:301–307.
54 Taub DD, Murphy WJ, Longo DL: Rejuvenation of the aging thymus: growth hormone-mediated and ghrelin-mediated signaling pathways. Curr Opin Pharmacol;10:408–424.
55 Chen BJ, Cui X, Sempowski GD, Chao NJ: Growth hormone accelerates immune recovery following allogeneic T-cell-depleted bone marrow transplantation in mice. Exp Hematol 2003;31:953–958.
56 Smaniotto S, Ribeiro-Carvalho MM, Dardenne M, Savino W, de Mello-Coelho V: Growth hormone stimulates the selective trafficking of thymic CD4+CD8– emigrants to peripheral lymphoid organs. Neuroimmunomodulation 2004;11:299–306.
57 Savino W, Smaniotto S, De Mello-Coelho V, Dardenne M: Is there a role for growth hormone upon intrathymic T-cell migration? Ann NY Acad Sci 2000;917:748–754.
58 Savino W, Mendes-da-Cruz DA, Silva JS, Dardenne M, Cotta-de-Almeida V: Intrathymic T-cell migration: a combinatorial interplay of extracellular matrix and chemokines? Trends Immunol 2002;23:305–313.
59 Knyszynski A, Adler-Kunin S, Globerson A: Effects of growth hormone on thymocyte development from progenitor cells in the bone marrow. Brain Behav Immun 1992;6:327–340.
60 Redelman D, Welniak LA, Taub D, Murphy WJ: Neuroendocrine hormones such as growth hormone and prolactin are integral members of the immunological cytokine network. Cell Immunol 2008;252:111–121.