

# Insulin-like growth factor-binding protein-4 inhibits growth of the thymus in transgenic mice

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## Abstract

Numerous *in vitro* studies have demonstrated that IGF-binding protein (IGFBP)-4 is a consistent inhibitor of IGF actions. In order to investigate the functions of IGFBP-4 *in vivo*, transgenic mice were generated by microinjection of a transgene, in which the murine *Igfbp4* cDNA is driven by the H-2K<sup>b</sup> promoter, and followed by a splicing cassette and polyadenylation signal of the human  $\beta$ -globin gene. Transgene mRNA was expressed ubiquitously, and elevated IGFBP-4 protein was detected in the spleen, thymus, kidney and lung of transgenic mice. The activities of serum IGFBPs were not changed in transgenic mice. Immunohistochemical studies revealed transgene expression predominantly in the thymic medulla and red pulp of the spleen. Body weight and the weights of the spleen, kidney and lung of transgenic mice were not different from controls. In contrast, the thymus of transgenic mice showed a significantly reduced weight and cortex volume. In transgenic thymus and spleen, cell proliferation was inhibited and apoptosis was stimulated. Transgenic mice showed normal T- and B-cell development and normal basal plasma immunoglobulin levels. In conclusion, overexpression of IGFBP-4 inhibits growth of the thymus. IGFBP-4 excess inhibits cell proliferation and stimulates apoptosis in lymphoid tissues, but does not affect lymphocyte development. These findings suggest that IGFBP-4 is a potential growth inhibitor of lymphoid tissues.

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## Introduction

Insulin-like growth factors-I and -II (IGFs-I and -II) are two important growth-promoting factors which regulate cellular survival, proliferation and differentiation (Stewart & Rotwein 1996). Most of these actions are mediated by the type-I IGF receptor (IGF-IR) and are regulated by the IGF-binding proteins (IGFBPs), which represent a family of six secreted proteins with a common domain organization (Hwa *et al.* 1999). Despite their structural similarity, each IGFBP has unique properties and exhibits specific functions. IGFBPs inhibit and/or potentiate IGF actions, and some of them have effects independent of IGF binding (Firth & Baxter 2002, Mohan & Baylink 2002).

Among the six IGFBPs, IGFBP-4 is the smallest one and exists in both non-glycosylated (24 kDa) and N-glycosylated (28 kDa) forms in all biological fluids. IGFBP-4 is unique among the six IGFBPs in having two extra cysteine residues in the variable central domain, which are linked by a disulfide linkage. IGFBP-4 is expressed by a wide range of cell types and tissues, and its expression is regulated by different mechanisms in a cell-type-specific manner (for review see Zhou *et al.* 2003).

IGFBP-4 was first described on the basis of its ability to potently inhibit bone cell growth (Mohan *et al.* 1989) and follicle-stimulating hormone (FSH)-stimulated steroid production of ovarian granulosa cells (Ui *et al.* 1989). It binds IGF-I and IGF-II with similar affinities and inhibits their

actions under most physiological and pathological conditions. IGFBP-4 inhibits IGF-induced cell proliferation and differentiation in many cell types (for review see Zhou *et al.* 2003). Local administration of recombinant IGFBP-4 inhibited IGF-I-induced increases of bone formation in mice (Miyakoshi *et al.* 1999). In contrast, systemic administration of IGFBP-4 stimulated bone formation, probably due to increasing IGF bioavailability via an IGFBP-4 protease-dependent mechanism (Miyakoshi *et al.* 2001). Transgenic mice overexpressing IGFBP-4 selectively in smooth muscle cells exhibited smooth muscle hypoplasia (Wang *et al.* 1998), which was in direct contrast to the smooth muscle hypertrophy induced by IGF-I overexpression (Wang *et al.* 1997). Moreover, a protease-resistant IGFBP-4 had more potency (Zhang *et al.* 2002), and IGF-I/IGFBP-4 double transgenic mice showed a reduction in wet weight of selected smooth muscle tissues (Wang *et al.* 1998), suggesting that these inhibitory effects of IGFBP-4 are IGF-I dependent.

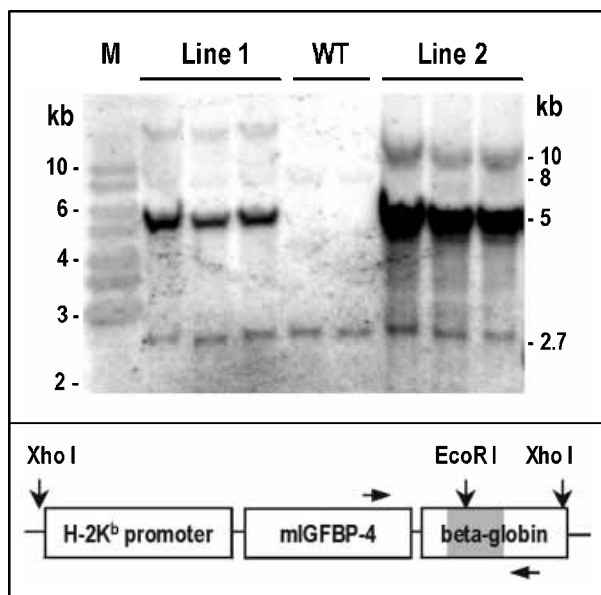
In addition, IGF-independent actions of IGFBP-4 have also been suggested (for review see Zhou *et al.* 2003). However, unlike IGFBPs-3 and -5, a specific receptor for IGFBP-4 has not been identified yet.

In order to investigate the function of IGFBP-4 *in vivo*, we generated transgenic mice overexpressing murine IGFBP-4 under the control of the murine H-2K<sup>b</sup> promoter. The latter was isolated from a class I gene of the major histocompatibility complex and shown to facilitate ubiquitous expression of transgenes with high levels in lymphoid organs (Morello *et al.* 1986). As the IGFBP-4 transgene was highly expressed in the spleen and thymus, we focused on the characterization of effects on growth and development of lymphoid organs.

## Materials and methods

### Construction and transfection of pH-2K<sup>b</sup>-mIGFBP-4

The mouse *Igfbp4* complementary DNA (kindly donated by Dr Drop, University of Rotterdam, The Netherlands) was released from pGEM4Z-mIGFBP-4, blunt ended and cloned into the blunted SalI restriction site of the mammalian expression vector pUC-H2 XXS (Plitz *et al.* 1999).



**Figure 1** Upper panel, identification of IGFBP-4 transgenic lines by Southern blot analysis. Ten micrograms of genomic DNA of two wild-type (WT) mice and three transgenic mice from the two transgenic lines were digested with EcoRI and analyzed by Southern blot hybridization, using an  $\alpha$ -[<sup>32</sup>P]dCTP-labeled IGFBP-4 cDNA probe. A kilobase DNA ladder (M) was loaded as the molecular weight standard. Lower panel, schematic representation of the H-2K<sup>b</sup>-mIGFBP-4 construct. The XhoI microinjection fragment (5 kb) contains the H-2K<sup>b</sup> promoter (2.1 kb), the murine IGFBP-4 cDNA (mIGFBP-4, 1176 bp), and a 1.7 kb human  $\beta$ -globin splice cassette (beta-globin) including the last 20 bp of exon 2, all of intron 2 (850 bp; shaded box) and exon 3 (228 bp) and the polyadenylation signal sequence (593 bp). Transgene-specific PCR primers are indicated by arrows.

The orientation of the insert in the sense direction was confirmed by restriction enzyme digest. The construct pH-2K<sup>b</sup>-mIGFBP-4 (Fig. 1) was tested by transient transfection of murine NIH-3T3 cells (ATCC, Manassas, VA, USA; 60% confluent in a six-well plate) with 4  $\mu$ g pH-2K<sup>b</sup>-mIGFBP-4 or the mock vector pUC-H2 XXS using SuperFect transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Transfected monolayers were washed once with PBS (pH 7.4), kept in the culture medium (Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum; Gibco) for 48 h, and then washed twice with PBS and incubated with 2 ml serum-free medium for another 48 h. The serum-free conditioned media (SFCM)

were analyzed by Western ligand and Western immunoblot.

### Generation of transgenic mice

All mice were purchased from Elevage Janvier (Le Genest-Saint-Isle, France) and Charles River Laboratories (Sulzfeld, Germany), and were maintained and bred under specific pathogen-free conditions according to the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA). Transgenic mice were generated by microinjection of the 5 kb XhoI fragment released from the expression vector pH-2K<sup>b</sup>-mIGFBP-4 into pronuclei of F2 zygotes from B6D2F1 (C57BL/6 × DBA/2) parents (Charles River Laboratories). The microinjected zygotes were implanted into the oviducts of pseudopregnant Naval Medical Research Institute (NMRI) mice (Charles River Laboratories) and carried to term. Founder transgenic mice were backcrossed with wild-type C57BL/6 mice (Elevage Janvier) for establishment and propagation of lines. Transgenic mice were identified by PCR (Taq DNA polymerase; Qiagen, Hilden, Germany) using DNA from tail clips. The tail tips of 3-week-old mice were lysed overnight in Kawasaki buffer (20 mM Tris-HCl (pH 7.5), 1.5 mM MgCl<sub>2</sub>, 25 mM KCl, 0.5% (v/v) Tween 20) containing 1.2 mg/ml proteinase K at 56 °C. The samples were then heated at 95 °C for 15 min to inactivate the proteinase K, and centrifuged at 15 300 *g* and 4 °C for 5 min; 2 µl of the supernatant were used for PCR analysis. The integrity of the genomic DNA was confirmed by amplifying a sequence of the housekeeping gene β-actin as described previously (Schneider *et al.* 2001). The integration of the construct was determined by PCR using sense primer mBP4#14 within the *Igfbp4* cDNA (5'-TAA GCC TGA GCC TTC TCG TG-3') and antisense primer β-globin#1 within the downstream exon of the β-globin splice cassette (5'-GGC AGC CTG CAC TGG TGG-3'). Both PCR amplifications were performed as follows: 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min. After a final 10 min extension at 72 °C the amplified products were separated in 1.2% agarose gels and visualized by ethidium bromide staining under u.v. light. The transgene integration pattern was analyzed by Southern blot

as described previously (Hoefflich *et al.* 1999) using an [ $\alpha$ -<sup>32</sup>P]dCTP-labeled mouse IGFBP-4 cDNA probe. Genomic DNA was isolated from tail biopsies using the Wizard genomic DNA purification kit (Promega) and hydrolysed with EcoRI. Transgene expression was determined by reverse transcription (RT)-PCR, Western ligand and Western immunoblot analyses.

### RT-PCR

The human β-globin splicing cassette in the transgene construct allows the differentiation of transgene-specific transcripts from the endogenous IGFBP-4 mRNA by RT-PCR. Total RNA was isolated from the cell cultures and different mouse tissues using the TriPure isolation reagent (Roche) according to the manufacturer's instructions. RT was performed as described previously (Schneider *et al.* 2001). PCR amplification was carried out using the transgene-specific primers mBP4#14 and β-globin#1 and the β-actin primers as described above. Control PCR reactions omitting RT and cDNA were also carried out.

### Western ligand blot

Tissue samples (20 mg) were homogenized in 0.5 ml protein extraction buffer (20 mM Tris-HCl (pH 7.5) and 2% (v/v) Triton X-100 in Laemmli buffer) using a cell homogenizer (ART, Muehlheim, Germany); 50 µg of protein from the tissue extracts, 1 µl serum or 16 µl SFCM were diluted in Laemmli buffer, boiled for 5 min, separated on 5% stacking/15% separating SDS-polyacrylamide gels, and electroblotted to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Western ligand blotting was carried out as described previously using an <sup>125</sup>I-labeled recombinant human IGF-II tracer (Schneider *et al.* 2001).

### Western blot

Blots were prepared as above, washed once in TBS-T (20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.1% Tween-20) at room temperature for 10 min and blocked at 4 °C overnight with blocking solution (TBS-T containing 3% (w/v) fat-free

milk powder). Thereafter the blots were incubated at room temperature for 60 min with the rabbit anti-IGFBP-4 polyclonal antiserum (Upstate Biotechnology, Lake Placid, NY, USA) diluted 1:1000 in blocking solution, and then washed three times in TBS-T. Secondary antibody (peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG); Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was diluted 1:5000 in blocking solution, and incubated with the blots at room temperature for 1 h. Finally, the blots were washed three to five times in TBS-T. Peroxidase (POD) activity was detected with the ECL Western blotting detection reagent (Amersham), and visualized with Kodak Digital Image Station (NEN Life Science Products, Inc., Belgium).

### Immunohistochemistry

The spleen and thymus were fixed in 4% PBS-buffered paraformaldehyde (pH 7.4) for 18–24 h, dehydrated in an ascending ethanol series and embedded in paraffin. Sections (5  $\mu$ m) were deparaffinized in xylene and hydrated in a descending ethanol series to distilled water. Antigen retrieval was performed by microwave irradiation in 10 mM citrate buffer (pH 6.0), for 2  $\times$  8 min at 750 W, followed by cooling to room temperature and washing twice in distilled water. Endogenous POD activity was blocked in 1% H<sub>2</sub>O<sub>2</sub> in PBS for 15 min, followed by washing in PBS for 2  $\times$  5 min. Blocking solution (10% normal rabbit serum in PBS) was applied for 60 min at room temperature, followed by incubation with the primary antibody, goat anti-IGFBP-4 polyclonal antibody (C-20; Santa Cruz Biotechnology Inc., Heidelberg, Germany), overnight at 4 °C in a dark humid chamber (the primary antibody was diluted in the blocking solution: for spleen sections, 1:100; for thymus sections, 1:50). Negative controls were incubated with blocking solution without primary antibody. Thereafter, the sections were washed in PBS for 3  $\times$  5 min. The second antibody, POD-conjugated rabbit anti-goat immunoglobulins (DakoCytomation GmbH, Hamburg, Germany), was diluted 1:100 and applied to each section for 60 min at room temperature. Sections were washed in PBS for 3  $\times$  5 min and developed with 0.05% 3,3'-diaminobenzidine (DAB) and 0.03% H<sub>2</sub>O<sub>2</sub> in PBS for 5 min, followed by counterstaining with hematoxylin.

### Determination of body and organ weights

The body weight of mice was recorded once a week from 1 to 12 weeks of age. Mice from selected groups (at 4, 8 and 12 weeks), were anesthetized with ether, weighed and bled from the retro-orbital sinus. After measurement of the distance between the nose and the base of the tail (nose–rump length, NRL), the mice were killed by cervical dislocation. Organs and carcass were dissected, blotted dry and weighed. Complete organs or parts of them were frozen on dry ice and later stored at –80 °C for RNA and protein isolation, or fixed in 4% paraformaldehyde for histological analysis.

### Histomorphometry

The thymus and spleen were released from the surrounding tissues and weighed to the nearest 0.1 mg. Their volumes were determined by the fluid displacement method according to Scherle (1970). The organs were fixed in 4% paraformaldehyde and embedded in paraffin. Cavalieri's principle (Gundersen & Jensen 1987) was applied to estimate the volumes of the paraffin-embedded thymus and spleen. First, the embedded organ was trimmed free of paraffin, and its length along the longitudinal axis was recorded. After positioning the first cut randomly within an interval of 1 mm, the organs were exhaustively sectioned perpendicular to their longitudinal axes into parallel slices of approximately 1 mm. These slices were placed with the right-hand cut-surface upward in tissue capsules and re-embedded in paraffin. Sections of 5  $\mu$ m thickness representing systematic samples of the whole organs were prepared and stained with hematoxylin and eosin (HE). Light-microscopic planimetric evaluation was performed on a semiautomated image analysis system (Videoplan; Zeiss-Kontron, Eching, Germany) coupled to a microscope via a color video camera (Panasonic, Japan). A 2.5  $\times$  objective was used, providing a 90  $\times$  final linear magnification. An object micrometer (Zeiss) was used for calibration. The cross-sectional areas of organ structures of interest were determined on images displayed on a color monitor. These structures included the thymus and its cortex and medulla; and the spleen and its red pulp and white pulp, including the marginal zone. A correction factor for shrinkage due to histological processing was calculated for each organ as the

volume of the unfixed organ divided by the volume of the embedded organ. Assuming the same extent of shrinkage for the whole organ and its different compartments, the volumes of the different parts of the whole organ were calculated taking the individual shrinkage into account.

### Flow cytometry

Blood (400–600  $\mu$ l) was collected by bleeding from the retro-orbital sinus in a 1 ml lithium–heparin tube (Kabe Labortechnik GmbH, Nuembrecht-Elsenroth, Germany), centrifuged at 4500 *g* for 5 min, and the plasma was recovered for ELISA analysis. The nucleated cells were prepared as described previously (Flaswinkel *et al.* 2000). Thymus, spleen and subiliac lymph nodes were cut into small pieces in FACS buffer (PBS containing 2% FCS and 0.01%  $\text{NaN}_3$ ) and were pushed through a 100  $\mu$ m nylon cell strainer (Becton Dickinson Labware, Le Pont de Claix, France) to obtain single-cell suspensions. Subsequently, thymocytes and cells from the lymph node were washed twice in FACS buffer. Erythrocytes in the single-cell suspensions from spleen were removed by incubating in 10 ml lysis buffer (140 mM  $\text{NH}_4\text{Cl}$ , 17 mM Tris–HCl (pH 7.2)) for 15 min, followed by two washing steps in FACS buffer. Suspensions of bone marrow cells were flushed from the tibia with FACS buffer. Immunofluorescence staining and subsequent measurement as well as data analysis were performed as described previously (Flaswinkel *et al.* 2000).

### *In vitro* proliferation assay

Splenocytes were cultured in flat-bottomed, 96-well polystyrene microtiter plates (Nunc, Kamstrup, Denmark) at a density of  $2 \times 10^5$  cells/ml in 200  $\mu$ l RPMI 1640 medium (Gibco) supplemented with 50  $\mu$ M 2-mercaptoethanol (Sigma), 4 mM glutamine, 1 mM pyruvate, 10% heat-inactivated FCS, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (Gibco). Splenocytes were stimulated either with 2  $\mu$ g/ml concanavalin A (Con A; Sigma) or 10  $\mu$ g/ml lipopolysaccharide (LPS; Sigma). After 48 h of culture at 37 °C in an atmosphere of 5%  $\text{CO}_2$  and 100% relative humidity, the cells were pulsed with 1  $\mu$ Ci [ $^3\text{H}$ ]thymidine (Amersham) per

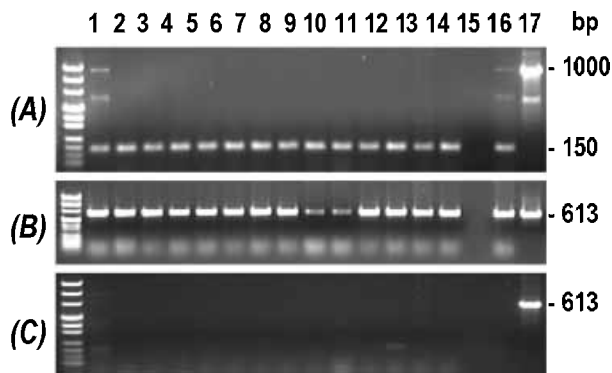
well for an additional 18 h of culture. The cells were harvested using a Micro 96 harvester (Skatron Instruments, Tranby, Norway). Incorporation of [ $^3\text{H}$ ]thymidine was quantified in a Betaplate liquid scintillation counter (Wallac, Gaithersburg, MD, USA).

### *In situ* proliferation assay

Two transgenic and two wild-type mice were injected intraperitoneally with 5-bromodeoxyuridine (BrdU; 30  $\mu$ g/g body weight). The animals were killed 2 h later. Spleen and thymus were fixed in 70% ethanol and embedded in paraffin. Sections (5  $\mu$ m) were prepared by standard procedures. Endogenous POD activity was blocked as described above, followed by washing in PBS. Then the sections were incubated in the following solutions separately: 30 min in 2 M HCl, 1 min in PBS,  $2 \times 10$  min in 70% ethanol/0.1 M Tris–HCl (pH 7.5),  $2 \times 5$  min in 70% ethanol, shortly in distilled water and  $2 \times 10$  min in PBS. POD-conjugated anti-BrdU antibody (1:10; Roche) was added to the sections and incubated overnight at 4 °C in a dark humid chamber. Sections were washed in PBS, developed with DAB, counterstained with hematoxylin, and scored under a light microscope (Zeiss Axiovert 200) coupled to a color video camera (Zeiss AxioCam). Photomicrographs were taken and edited using Zeiss Axioversion Image software. The proliferation index (PI) was calculated from the percentage of BrdU-positive cells counted in the red pulp and white pulp of spleen, and in the cortex and medulla of thymus. Five random fields (50  $\mu$ m  $\times$  50  $\mu$ m, about 100 cells) in the red pulp and white pulp, and cortex and medulla were counted.

### *In situ* apoptosis assay

The spleen and thymus from two transgenic and two wild-type mice were fixed in 4% paraformaldehyde, routinely processed and embedded in paraffin. Sections of 5  $\mu$ m thickness were prepared, deparaffinized by standard procedures, treated with 750 W microwave irradiation for 1 min in 0.1 M citrate buffer (pH 6.0), cooled to room temperature and rinsed in PBS. Endogenous POD activity was blocked as described above, followed by washing in PBS. The terminal deoxynucleotide transferase



**Figure 2** RT-PCR analysis of transgene mRNA expression in different tissues of transgenic mice: adrenal gland (1), bladder (2), brain (3), heart (4), kidney (5), liver (6), lung (7), skeletal muscle (8), ovary (9), salivary gland (10), skin (11), spleen (12), testis (13) and thymus (14). 15, water; 16, IGFBP-4-transfected cells. 17, plasmids pH-2K<sup>b</sup>-mIGFBP-4 (A) or pCR-TOP-β-actin (B and C). (A) RT-PCR using primers mBP4#14 and β-globin#1. (B) β-actin RT-PCR. (C) control PCR omitting RT using β-actin primers.

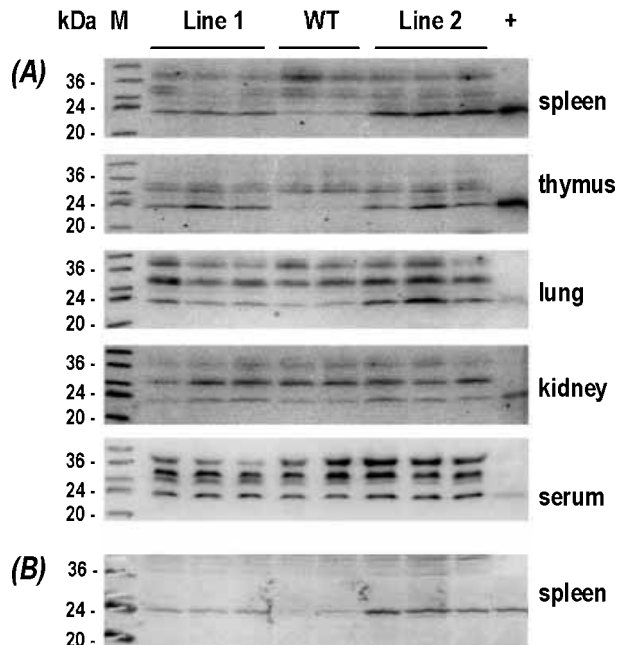
(TdT)-mediated dUTP nick end labeling (TUNEL) was carried out using the *In Situ* Cell Death detection kit (Roche). Each experiment was performed with a negative control (labeling solution without TdT). Fluorescein-labeled dNTPs were then detected by incubating sections with converter-POD (POD-conjugated anti-fluorescein antibody; Roche) at 37 °C for 30 min in a dark humid chamber. Sections were washed in PBS, developed with DAB and counterstained with hematoxylin. The apoptosis index (AI) was calculated from the percentage of TUNEL-positive cells counted in the red pulp and white pulp of spleen, and in the cortex and medulla of thymus as described above.

### Basal immunoglobulin ELISA

Basal immunoglobulin levels in plasma were determined by using sandwich ELISAs specific for IgM, IgG3 and IgA as described previously (Flaswinkel *et al.* 2000).

### Autoreactive antibody assay

Plasma anti-DNA autoreactive antibody levels were determined using the ELISA-based assay as described previously (Flaswinkel *et al.* 2000).



**Figure 3** <sup>125</sup>I-labeled IGF-II ligand blot (A) and Western immunoblot (B) analysis of IGFBP-4 expression in transgenic and control (WT) mice. IGFBP-4 levels were increased in the spleen, thymus, lung and kidney. No significant change was found in serum for each IGFBP. The serum-free conditioned media from mIGFBP-4-transfected cells were loaded as IGFBP-4 protein-positive control (+).

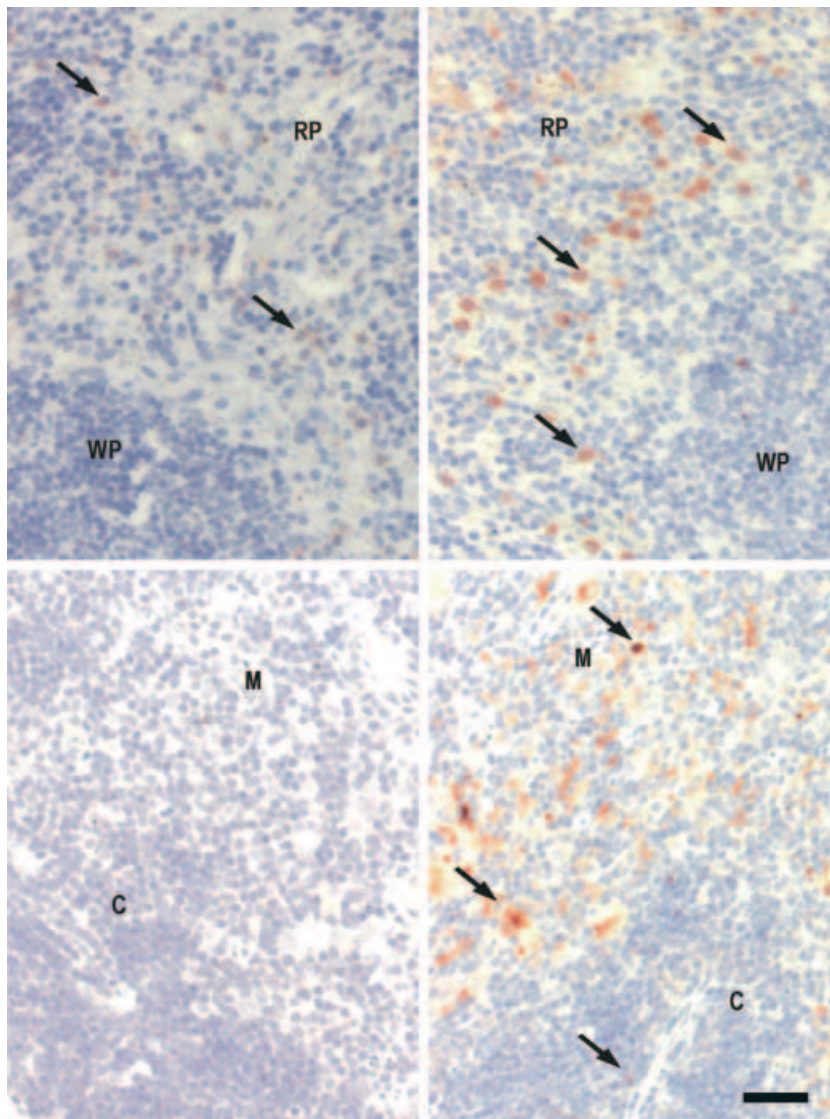
### Statistical analysis

The data were analyzed for significance of differences using Student's *t*-test. A difference was considered to be statistically significant at  $P < 0.05$ .

## Results

### Generation of IGFBP-4 transgenic mice and examination of transgene expression

When conditioned media of NIH-3T3 cells transiently transfected with pH-2K<sup>b</sup>-mIGFBP-4 and mock vector were analyzed by <sup>125</sup>I-labeled IGF-II ligand blot, the level of a 24 kDa protein was significantly increased in the media from pH-2K<sup>b</sup>-mIGFBP-4 transfected cells, compared with the media from the parental cells and mock transfected cells (data not shown). This protein was identified as being IGFBP-4 by Western blot using IGFBP-4 antibodies.

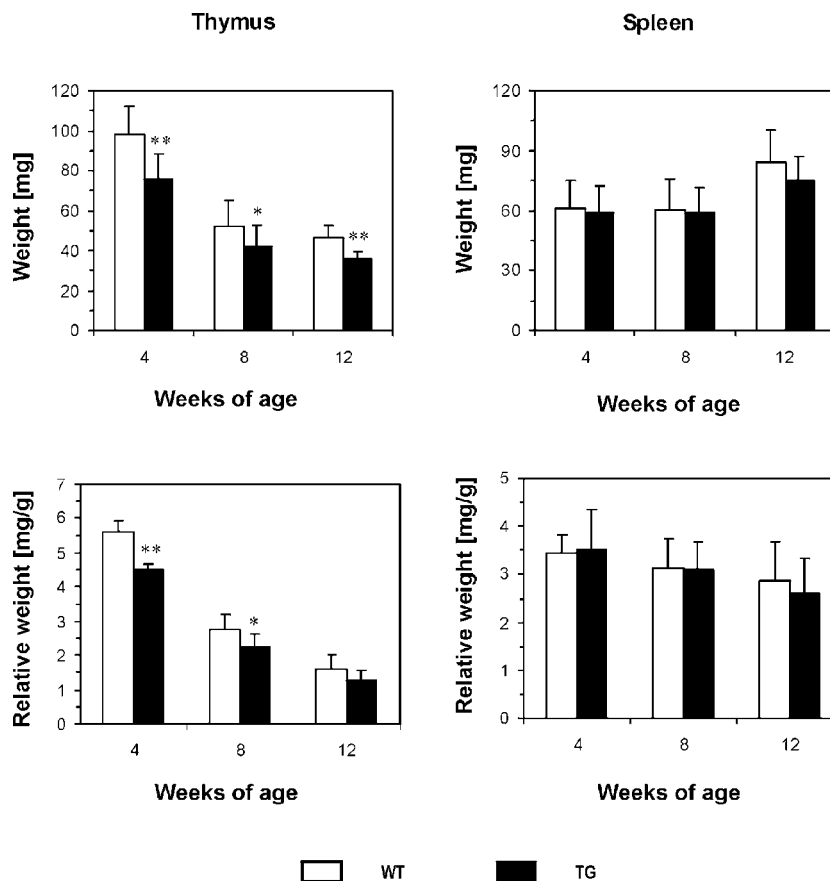


**Figure 4** IGFBP-4 immunostaining of the spleen (upper panels) and thymus (lower panels) of wild-type (left) and transgenic mice (right). Strong signals were detected in the red pulp of the spleen (RP) and medulla of the thymus (M) of transgenic mice. WP, white pulp; C, cortex. Scale bar, 20  $\mu$ m.

Two founder mice were obtained from the microinjection experiments and were mated with wild-type C57BL/6 mice, generating two transgenic lines. There were more transgene copies integrated in the genome of line 2 than line 1, as estimated by Southern blot analysis (Fig. 1). The endogenous *Igfbp4* signals were detected at 8 kb and 2.7 kb in all wild-type and transgenic animals. The 5 kb microinjection fragment was recovered in both transgenic lines, and the line-specific signal was

found at 10 kb in line 2 and >10 kb in line 1, indicating different integration sites in the two transgenic lines.

RT-PCR revealed ubiquitous expression of the transgene in all organs/tissues tested, including adrenal gland, bladder, brain, heart, kidney, liver, lung, ovary/testis, salivary gland, skeletal muscle, skin, spleen, stomach, small and large intestine, and thymus (Fig. 2). However, elevated IGF-binding activity was only detected in the spleen, thymus,



**Figure 5** Absolute and relative (relative to body weight) weights of thymus (left) and spleen (right) in transgenic mice (TG) and their wild-type littermates (WT). From each group, 15–20 mice were investigated. Data are presented as means  $\pm$  s.d. \* $P < 0.05$ , \*\* $P < 0.01$ .

lung and kidney as shown by IGF-II ligand blot (Fig. 3A). The increased IGFBP at 24 kDa in the spleen of transgenic mice was confirmed to be IGFBP-4 by Western blot using IGFBP-4-specific antibodies (Fig. 3B). IGFBP-4 expression was consistently higher in line 2 than in line 1. The activities of serum IGFbps were not changed in transgenic mice as shown by Western ligand blot (Fig. 3A).

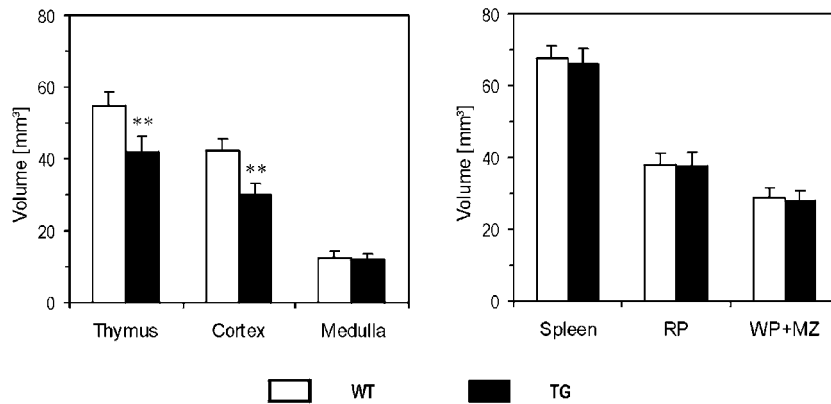
In order to localize IGFBP-4 expression in the spleen and thymus, immunohistochemical staining was performed. Strong IGFBP-4 immunoreactivity was detected selectively in the splenic red pulp and thymic medulla of transgenic mice. The predominant IGFBP-4 positive cells were macrophages in the red pulp of the spleen and epithelial cells in the thymus medulla (Fig. 4).

### Reduced thymus size in IGFBP-4 transgenic mice

Transgenic mice and non-transgenic littermates from both lines were weighed once a week from 1 to 12 weeks of age to detect the possible effect of transgene expression on body weight gain. No significant difference was observed between transgenic and control animals from both lines (data not shown).

NRL, body and organ weights were recorded in 4-, 8- and 12-week-old animals. There were also no significant differences in NRL, body and organ weights between both groups, with the exception of thymus weight, which was significantly reduced in transgenic mice when compared with controls. This reduction persisted when thymus weight was





**Figure 6** Morphometric determination of the volumes of thymus, spleen and their compartments of 8-week-old transgenic (TG) and control (WT) mice. Four animals from each group were investigated as described in the Materials and methods section. Data are presented as means $\pm$ s.d. \*\*\* $P$ <0.01.

related to body weight (Fig. 5). Morphometric analysis revealed that the volumes of thymus and its cortex were significantly reduced in transgenic animals compared with control littermates, whereas the volume of thymic medulla was not different between the two groups (Fig. 6). In contrast, there were no significant differences in weight (Fig. 5), total and compartmental volumes of spleen between the two groups (Fig. 6).

### Decreased cell proliferation and increased apoptosis in the spleen and thymus of transgenic mice

To analyze the proliferative capacity of T- and B-cells of the transgenic mice, splenocytes were stimulated *in vitro* with mitogens Con A and LPS respectively. [ $^3$ H]thymidine incorporation experiments revealed that the proliferative activity of the transgenic splenocytes was significantly reduced after Con A ( $P$ <0.05) and LPS ( $P$ <0.01) stimulation (Fig. 7).

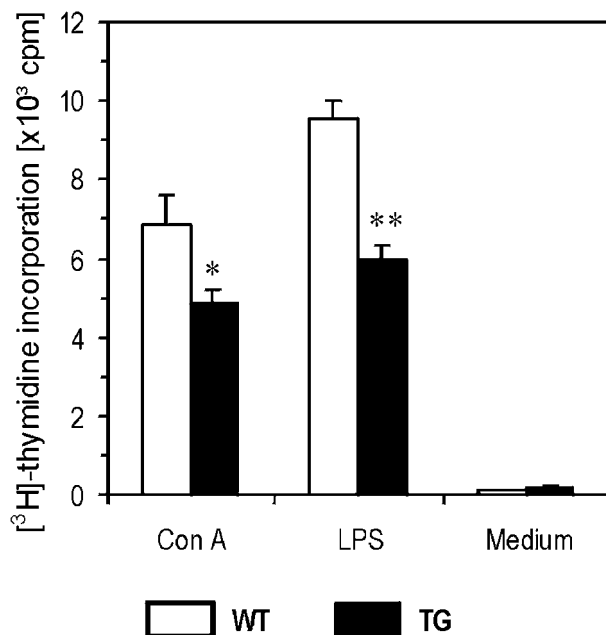
To visualize the proliferative status in the spleen and thymus, mice were injected 2 h before death with BrdU solution. As shown in Fig. 8, BrdU localized to the nuclei of proliferating cells, which were located predominantly in the cortex of thymus and in the red pulp of spleen (Fig. 8). The PI of the red pulp in the transgenic spleen was significantly decreased to  $4.7 \pm 3.0\%$  compared with  $8.6 \pm 1.2\%$  in control animals ( $P$ <0.05), and that of thymic cortex was decreased to  $3.9 \pm 2.8\%$  compared with  $12.9 \pm 2.7\%$  in the controls

( $P$ <0.001), while the PIs of splenic white pulp and thymic medulla were not different between transgenic and control mice.

We then performed TUNEL analysis of tissue sections of spleen and thymus to detect cells carrying fragmented DNA characteristic for apoptotic cells. TUNEL-positive cells located predominantly in the splenic red pulp and thymic cortex (Fig. 9). The AI of the red pulp in transgenic mice was significantly increased to  $6.4 \pm 3.2\%$  compared with  $2.2 \pm 1.1\%$  in control animals ( $P$ <0.05), and that of thymic cortex was increased to  $20.2 \pm 6.4\%$  compared with  $12.9 \pm 2.7\%$  in the controls ( $P$ <0.01), while the AIs of splenic white pulp and thymic medulla were not significantly different between the two groups.

### Normal lymphocyte development

To investigate the potential effect of transgene expression in the thymus and spleen on the lymphocyte development, the lymphocytes from the thymus, bone marrow, spleen, lymph node and peripheral blood of transgenic and control mice were analyzed by flow cytometry, using antibodies against different markers expressed on T- and B-cells and other populations of lymphocytes. FACS analysis did not reveal any significant differences in percentages of different cell populations from the primary and secondary immune organs (Fig. 10) as well as in peripheral blood (data not shown).



**Figure 7** *In vitro* proliferation assay. Splenocytes from 4-week-old transgenic (TG) mice ( $n=3$ ) and their wild-type (WT) littermates ( $n=3$ ) were cultured in 96-well microtiter plates at a density of  $2 \times 10^5$  cells/ml and were stimulated with Con A or LPS. Incorporation of [<sup>3</sup>H]thymidine was quantified as described in the Materials and methods section. Data are presented as means  $\pm$  S.D. \* $P < 0.05$ , \*\* $P < 0.01$ .

### Normal plasma immunoglobulin and autoreactive antibody levels

To detect possible effects of transgene expression on the production of the basal immunoglobulins and autoreactive antibodies, the plasma levels of IgM, IgG3, IgA and anti-DNA autoreactive antibodies were determined. Their levels in transgenic mice were not different from those of wild-type littermates (Fig. 11).

### Discussion

The cross-talk between the endocrine and immune systems has been suggested by numerous studies (for reviews see: Blalock 1994, Madden & Felten 1995, Besedovsky & del Rey 1996). Cytokines, the soluble factors secreted by the immune cells, exert biological actions on the endocrine system (Silva *et al.* 1998), conversely, a variety of hormones and peptide growth factors have receptors in the tissues of the immune system and modulate immune

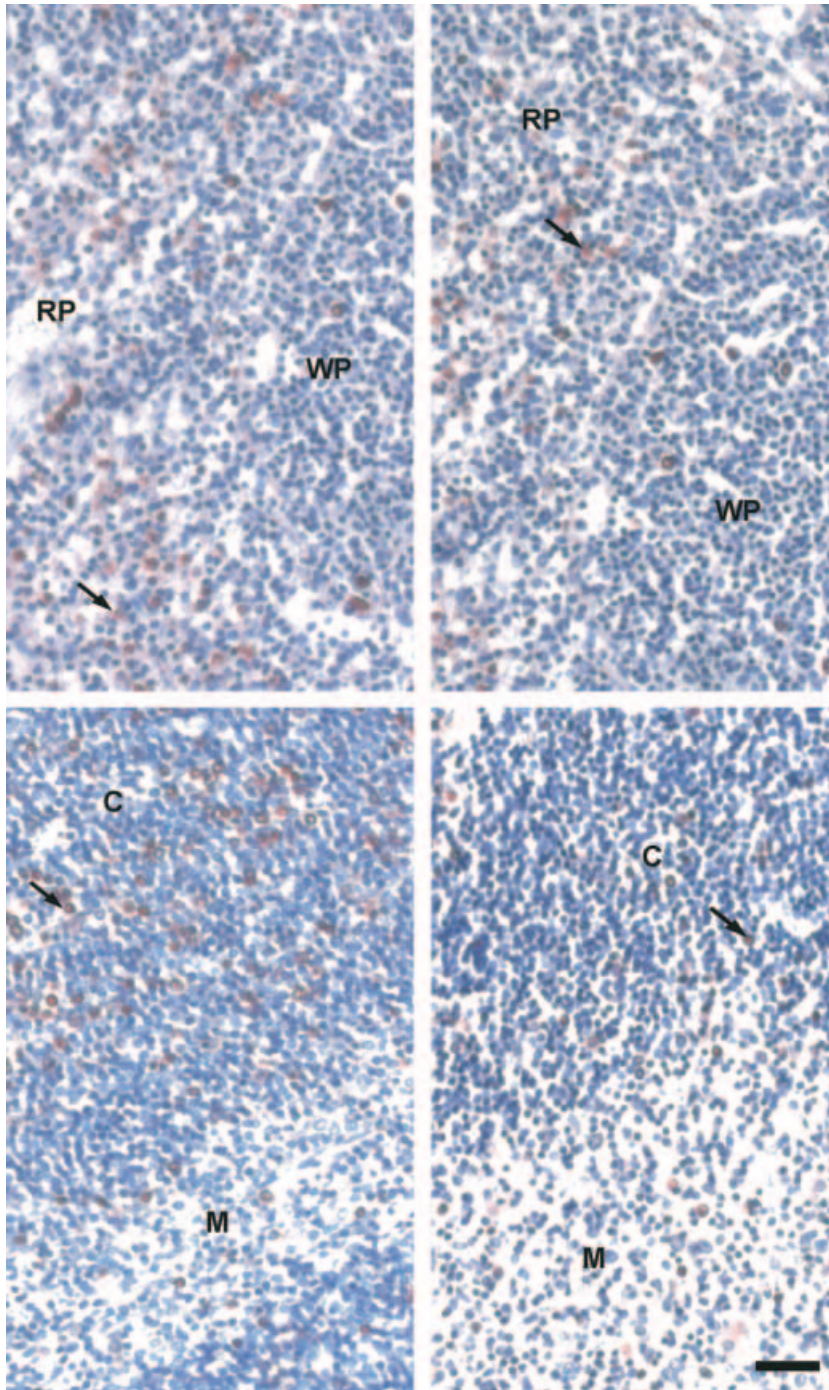
functions (Dorshkind & Horseman 2000). IGF-I and IGF-II are not only involved in endocrine modulation of the development and function of the immune system, but also act as cytokines to regulate local growth and differentiation (Buil-Offers & Kooijman 1998, Jeay *et al.* 2002). As important regulators, IGFBPs may also be involved in these processes. To investigate IGFBP-4 actions, we generated transgenic mice overexpressing IGFBP-4. As the transgene was highly expressed in the spleen and thymus, we had the opportunity to characterize the effects of IGFBP-4 excess on growth and development of these organs.

### Transgene expression

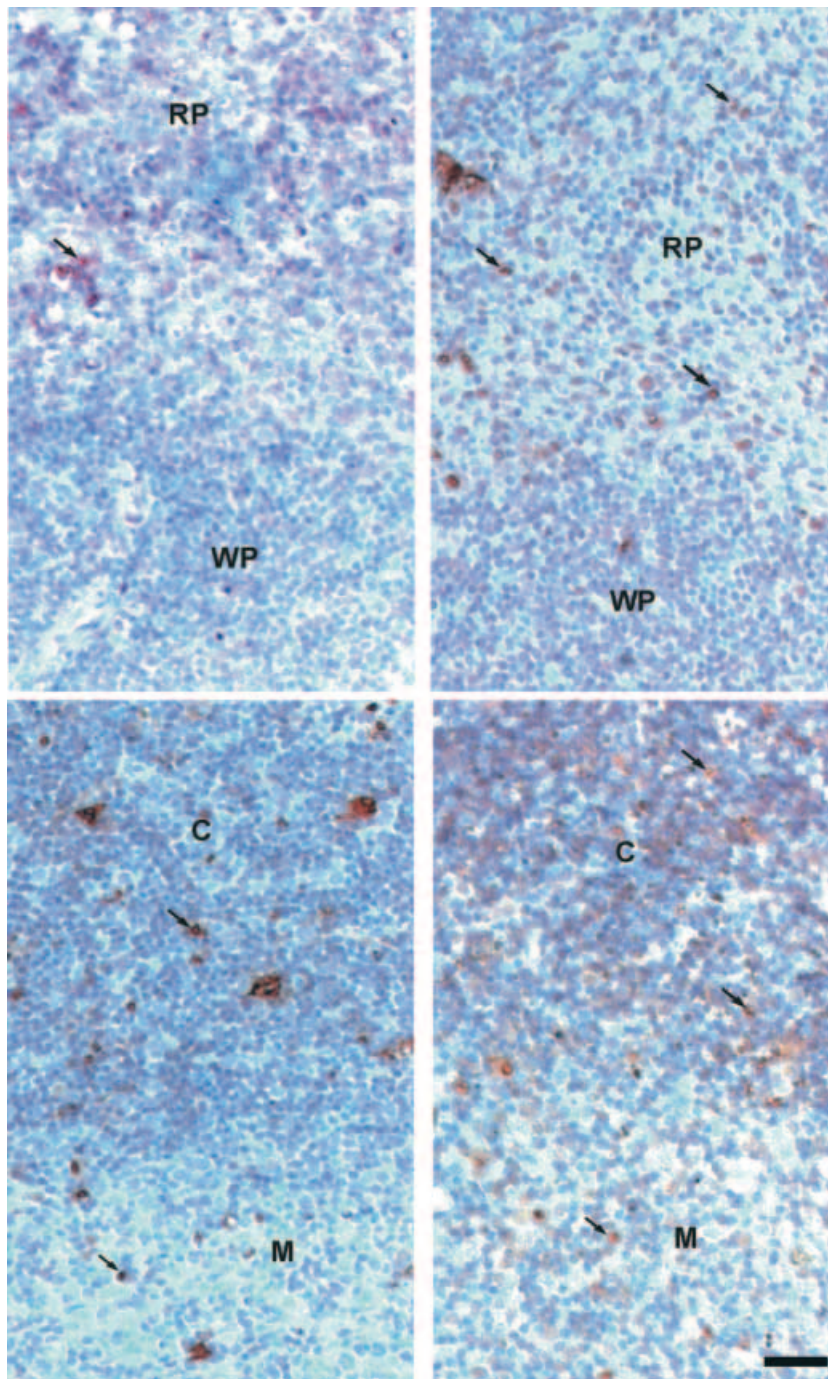
Transgenic mRNA was expressed ubiquitously in all tissues of transgenic mice (Fig. 2), and elevated protein levels were detected in the spleen, thymus, lung and kidney (Fig. 3). Immunohistochemical studies revealed that strong IGFBP-4 immunoreactivity was located selectively in the red pulp of the spleen and the medulla of the thymus. The majority of IGFBP-4-positive cells were macrophages in the spleen red pulp and epithelial cells in the thymus medulla (Fig. 4). This result is in agreement with the location of IGF-II expression in the H-2K<sup>b</sup>-hIGF-II transgenic mice (Van der Ven *et al.* 1997). The expression pattern of the transgene was identical in the two different transgenic lines, indicating that expression of the transgene occurs independently of the integration sites. Elevated IGFBP-4 protein levels were detected in the spleen, thymus, lung and kidney, corresponding to high expression of the H-2K<sup>b</sup> gene in these organs (Morello *et al.* 1986). The murine H-2K<sup>b</sup> promoter has been used by several investigators for generations of transgenic mice expressing human growth hormone (hGH) (Morello *et al.* 1986), c-myc (Morello *et al.* 1989), c-fos (Ruther *et al.* 1988), hIGF-II (Buil-Offers *et al.* 1995) and mutant forms of the tumor necrosis factor receptor p55 (TNFRp55) (Plitz *et al.* 1999). In these mice, a tissue specificity similar to the endogenous H-2K<sup>b</sup> mRNA expression was also observed.

### Effects on body and organ growth

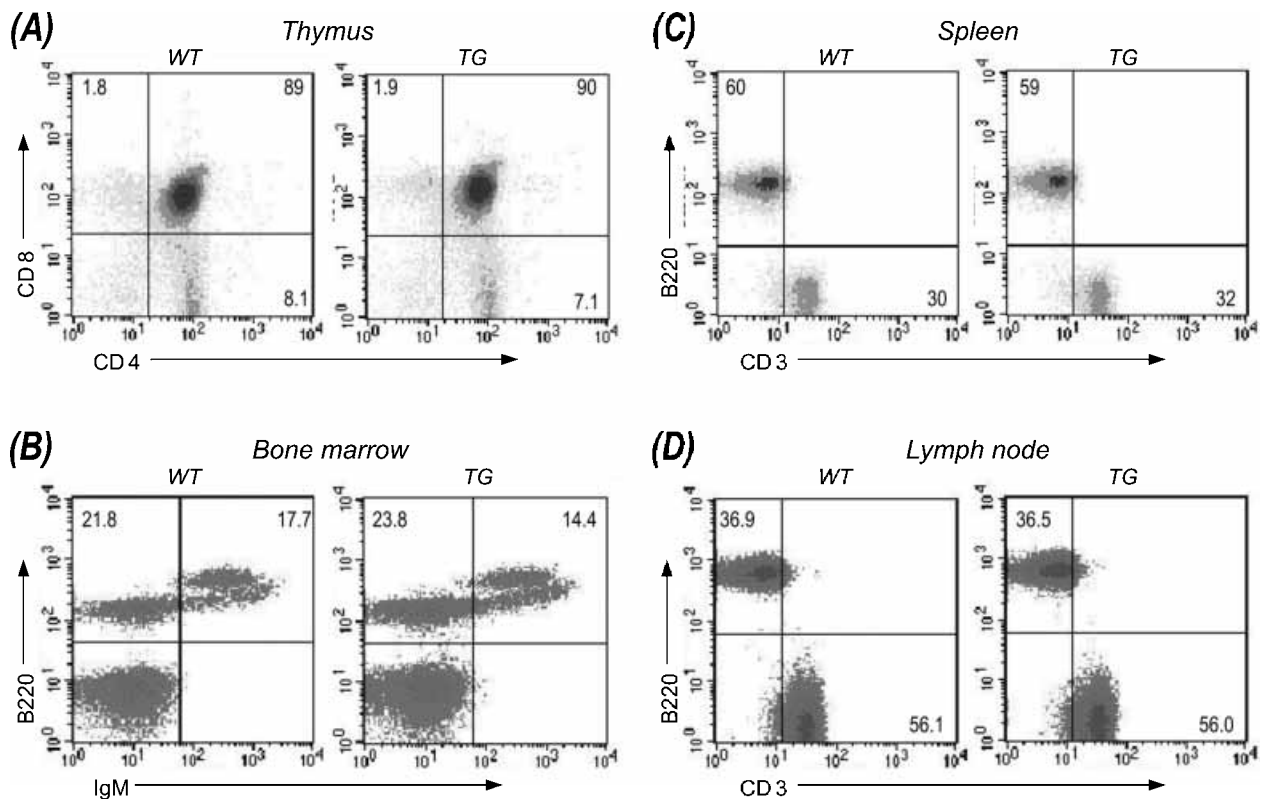
To investigate potential effects of transgene expression on body and organ growth, a large



**Figure 8** BrdU staining of the spleen (upper panels) and thymus (lower panels). Cell proliferation is documented by the presence of BrdU-positive cells (arrows), which are located predominantly in the red pulp (RP) and cortex (C). Note the decrease of proliferation rate in transgenic mice (right) compared with control mice (left). Scale bar, 20  $\mu$ m.



**Figure 9** TUNEL staining of the spleen (upper panels) and thymus (lower panels). Apoptotic cells (arrows) are located in the red pulp (RP) and cortex (C). Note the increase of apoptosis rate in transgenic mice (right) compared with wild-type mice (left). Scale bar, 20  $\mu$ m.



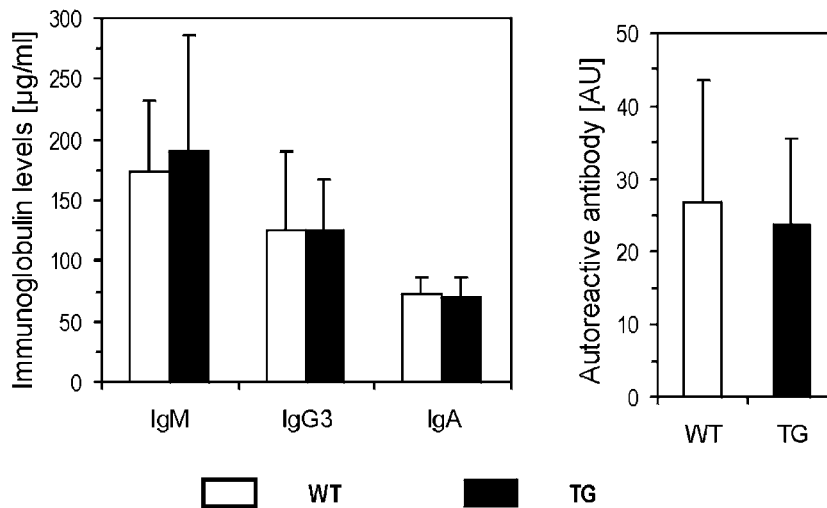
**Figure 10** Flow cytometric analysis of T- and B-cell development of 4-week-old transgenic (TG) and control (WT) mice. Three animals from each group were analysed. Density plots with characteristic stainings are shown. (A) Thymocyte development in the thymus from CD4/CD8 double-negative to CD4/CD8 double-positive and then to the CD4 or CD8 single-positive stages is not significantly different between TG and WT mice. (B) Development of B-cells in the bone marrow. Cells are gated on CD43<sup>-</sup>. Development proceeds comparably from pre-B cells (IgM<sup>-</sup>/B220<sup>+</sup>) to immature B-cells (IgM<sup>+</sup>/B220<sup>+</sup>) and finally to mature B-cells (IgM<sup>+</sup>/B220 high) in TG and WT mice. Secondary lymphoid organs are represented by spleen (C) and lymph node (D). In both cases expression of typical markers and distribution of T- and B-cells is not different between TG and WT mice.

panel of allometric measurements was carried out as described in the Materials and methods section.

The marked decrease in weight and volume of the thymus in the postnatal life is the outstanding feature of the H-2K<sup>b</sup>-IGFBP-4 transgenic mice (Fig. 5). Although the transgene was also expressed in the spleen, kidney and lung, the weights of these organs were not changed (data for kidney and lung were not shown), suggesting that IGFBP-4 over-expression does not affect postnatal growth of these organs. However, ultrastructural studies need to be done to clarify this point further.

In order to define the histological alterations in the thymus and spleen, histomorphometric analyses were performed. The transgenic mice showed a significant decrease in thymic cortex volume compared with the controls, whereas the volume of

the medulla was not altered (Fig. 6). IGFBP-4 was selectively expressed in the medulla of the thymus, but the histological effect was confined to the cortex. This finding is in agreement with the IGF-II effect in H-2K<sup>b</sup>-hIGF-II transgenic mice (Van der Ven *et al.* 1997), and may be due to a paracrine effect of IGFBP-4 and a relatively higher sensitivity of the cortical compartment to IGFBP-4. These notions were supported by the *in situ* proliferation and apoptosis studies, which revealed that proliferating and apoptotic cells were predominantly located in the cortex, and that cell proliferation was inhibited and apoptosis was stimulated selectively in the cortex of transgenic mice but not in the medulla (Figs 8 and 9). Although IGFBP-4 was also expressed in the spleen of transgenic mice, no alteration in the architecture of the spleen was



**Figure 11** Determination of the levels of plasma basal immunoglobulins and anti-DNA autoreactive antibodies. Ten animals from each group were investigated. No significant differences were detected between the two groups.

observed; this is in agreement with the unchanged weight and volume of this organ. Interestingly, H-2K<sup>b</sup>-hIGF-II transgenic mice did not show a spleen phenotype either (Buil-Offers *et al.* 1995, Van der Ven *et al.* 1997). hIGF-II was highly expressed in the spleen, thymus and liver of these mice, but only the postnatal growth of the thymus was promoted by IGF-II overexpression. The absence of splenomegaly in H-2K<sup>b</sup>-hIGF-II transgenic mice was suggested to be a consequence of upregulation of IGFBP-3 expression in the spleen by elevated IGF-II (Smink *et al.* 1999). Whether the expression of some growth-regulating genes was altered by IGFBP-4 overexpression in the spleen, lung and kidney of our transgenic mice is unknown. The exclusive effect of IGFBP-4 on the growth of the thymus might be the consequence of an interaction with a thymus-specific factor whose identity, however, remains to be unravelled.

### Effects on the immune functions

The functions of the IGF components in the immune system are very complex. Systemic administration of IGF-I significantly increased the size and cellularity of both primary and secondary lymphoid organs in rodents (Binz *et al.* 1990, Beschorner *et al.* 1991, Murphy *et al.* 1992, Clark *et al.* 1993, Jardieu *et al.* 1994). Furthermore, IGF-I

administration enhanced immune response and altered lymphocyte survival and regeneration in thymus and spleen of dexamethasone-treated rats (Hinton *et al.* 1995, 1998). Administration of IGF-II also stimulates growth of the thymus and spleen, but to a lesser extent than IGF-I (Buil-Offers *et al.* 1994, Conlon *et al.* 1995). Overexpression of IGF-I in mice stimulates T- and B-cell development and antigen-specific IgG synthesis (Clark *et al.* 1993, Robbins *et al.* 1994). Overexpression of IGF-II in transgenic mice stimulated the development of phenotypically normal T-cells but not mature B-cells (Kooijman *et al.* 1995, 1997). IGFbps are also expressed by lymphoid tissues. Normal human peripheral lymphocytes were shown to express mRNA for IGFBP-2 and -3, and after mitogenic stimulation they additionally express IGFBP-4 and -5 (Grellier *et al.* 1995). Thymic epithelial cells express different levels of IGFBP-2 to -6, with a predominance of IGFBP-4 (Kecha *et al.* 1999). IGFBP-4 has also been detected in murine thymic macrophages and in macrophage cell lines (Li *et al.* 1996). Overexpression of IGFBP-1 in transgenic mice led to inconsistent effects on spleen size (Dai *et al.* 1994, Murphy *et al.* 1995a, Rajkumar *et al.* 1995), overexpression of IGFBP-2 reduced spleen weight of male transgenic mice (Hoefflich *et al.* 1999), whereas overexpression of IGFBP-3 increased spleen size (Murphy *et al.* 1995b).

IGFBP-2 knockout mice show no gross phenotype except for a reduced spleen size (Wood *et al.* 2000).

Considering the high transgene expression in the spleen and thymus, we focused on the investigation of potential effects of IGFBP-4 overexpression on immune functions. First, the mitogenic response of T- and B-cells was determined by *in vitro* proliferation assays using T- and B-cell mitogens. The proliferative activity of the splenocytes was significantly reduced in transgenic mice after Con A and LPS stimulation (Fig. 7). To address the lymphocyte development of IGFBP-4 transgenic mice, FACS analysis was performed. No changes were found in percentages of different cell populations from the primary and secondary immune organs of transgenic mice (Fig. 10). This finding suggests that IGFBP-4 excess in the thymus and spleen did not affect the development of the immune-related cells. Finally, plasma levels of basal immunoglobulins and anti-DNA autoreactive antibodies were determined. No significant differences were found between transgenic and control animals (Fig. 11). These results indicate that IGFBP-4 excess in the thymus and spleen has no effect on the production of the humoral immunocompetence, and does not induce autoimmune disorders in transgenic mice.

In conclusion, overexpression of IGFBP-4 inhibits the growth of the thymus. IGFBP-4 excess inhibits cell proliferation and stimulates cell apoptosis in the lymphoid tissues, but does not affect lymphocytic development. These findings suggest that IGFBP-4 is a potential growth inhibitor of the lymphoid tissues.

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