Overexpression of Lymphotoxin in T Cells Induces Fulminant Thymic Involution

Mathias Heikenwalder,* Marco Prinz,* Nicolas Zeller,* Karl S. Lang,† Tobias Junt,‡ Simona Rossi,‖ Alexei Tumanov,‡ Hauke Schmidt,‖‖ Josef Priller,*,† Lukas Flatz,† Thomas Rülicke,** Andrew J. Macpherson,§ Georg A. Holländer,¶ Sergei A. Nedospasov,‡ and Adriano Aguzzi*†

From the Institutes of Neuropathology,* and Experimental Immunology,† University Hospital of Zürich, Zürich, Switzerland; the Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, and the Belozersky Institute of Physico-Chemical Biology,‡ Moscow State University, Moscow, Russia; the Department of Medicine,§ McMaster University, Hamilton, Ontario, Canada; Pediatric Immunology,‖ Department of Biomedicine, University of Basel, Basel, Switzerland; the Institute of Neuropathology and Neuropsychiatry,¶ Laboratory of Molecular Psychiatry, Charité-Universitätsmedizin Berlin, Berlin, Germany; the Institute of Laboratory Animal Science and Research Center Biomodels Austria,** University of Veterinary Medicine, Vienna, Austria; and the Institute for Neuropathology,¶¶ the Georg-August-University, Göttingen, Germany

Activated lymphocytes and lymphoid-tissue inducer cells express lymphotoxins (LTs), which are essential for the organogenesis and maintenance of lymphoid-tissue microenvironments. Here we describe that T-cell-restricted overexpression of LT induces fulminant thymic involution. This phenotype was prevented by ablation of the LT receptors tumor necrosis factor receptor (TNFR) 1 or LT beta receptor (LTβR), representing two non-redundant pathways. Multiple lines of transgenic LTBβ and LTα mice show such a phenotype, which was not observed on overexpression of LTβ alone. Reciprocal bone marrow transfers between LT-overexpressing and receptor-ablated mice show that involution was not due to a T cell-autonomous defect but was triggered by TNFR1 and LTβR signaling to radioreistant stromal cells. Thymic involution was partially prevented by the removal of one allele of LTβR but not of TNFR1, establishing a hierarchy in these signaling events. Infection with the lymphocytic choriomeningitis virus triggered a similar thymic pathology in wt, but not in Tnfr1−/− mice. These mice displayed elevated TNFα in both thymus and plasma, as well as increased LTs on both CD8+ and CD4+ CD8− thymocytes. These findings suggest that enhanced T cell-derived LT expression helps to control the physiological size of the thymic stroma and accelerates its involution via TNFR1/LTβR signaling in pathological conditions and possibly also in normal aging. (Am J Pathol 2008, 172:1555–1570; DOI: 10.2353/ajpath.2008.070572)

The efficient output of lymphocytes from the thymus following positive and negative selection is crucial for efficient adaptive immune responses. The thymus generates a T-cell repertoire essential for adaptive immunity during embryogenesis and throughout life. T-cell precursors generated from hematopoietic stem cells in the bone marrow (BM) enter the thymus under chemotactic guidance. A complex maturation process involving interactions between thymocytes and the thymic stroma ensures self-tolerance: most autoreactive thymocytes are eliminated by negative selection before exiting the thymus.

Impaired responses to pathogens and to altered self-antigens have been recognized as contributing to the aging of the immune system in many mammalian species. Age-dependent involution of the thymus parallels the age-related

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Current address of T.J.: Department of Pathology, University of Chicago, Chicago, IL. Current address of A.T.: Department of Neuropathology, University Hospital of Zürich, Zürich, Switzerland. E-mail: adriano.aguzzi@usz.ch.
deterioration of immune responses. In male C57BL/6 mice, thymic involution starts at the age of 8 to 12 weeks and results in a thymic rudiment after 6 to 8 months. In females this process is delayed. The key factors and cellular mechanisms driving involution are poorly understood.

Lymphotoxins (LTs) are expressed by activated T, B, and NK cells, as well as by CD4+CD3−CD4+CXCR5+ lymphoid tissue inducer cells (LTi cells). LTs and related tumor necrosis factor (TNF)-family cytokines are essential for the development and maintenance of secondary lymphoid organs and for appropriate immunological functions.

Membrane-bound LT consists of heterotrimeric complexes of LTα and LTβ (LTαβ or LTαβ), whereas secreted LT (sLTα) is homotrimeric. LT heterotrimers signal via LTβ receptor (LTβR), whereas sLTα signals mainly via the TNF receptors, TNFR1 and TNFR2. LTα is inducible in B and T cells, whereas LTβ mRNA is constitutively expressed. LT signaling is essential for the differentiation and function of high endothelial venules and ectopic LT expression can induce lymphoid neogenesis at atypical sites including kidney, pancreas, and liver. The cellular and molecular requirements for the establishment of lymph nodes and inflammation-associated neolymphoid structures are similar. In the latter, hyperactivated lymphocytes appear to be able to fulfill the role of LTi cells.

LT signaling deficiencies can lead to alymphoplasia, yet do not significantly prevent thymic development, thymic structure, T cell differentiation, and negative selection. However, LTβR signaling was found to control normal differentiation of thymic medullary epithelial cells via cross talk between thymocytes and thymic medullary epithelial cells, and to regulate γδ T cell differentiation in concert with other factors. LTβR plays an important role in thymic negative selection of organ-specific thymocytes through thymic medullary chemokine regulation. Thymic homeostasis and atrophy might be controlled or influenced by changes in homeostatic chemokines and cytokines. Administration of pro-inflammatory cytokines and steroids can drive thymic involution. LTβR induces lymphoid tissue abnormalities that affect the thymus. Enhanced levels of TNF after lipopolysaccharide or concanavalin A treatment may cause a similar phenotype, whereas ablation delays age-dependent thymic involution.

Naïve T cells do not express membrane-bound or soluble LTαβ yet up-regulate both forms on activation. LT receptors are expressed by thymic stroma, suggesting a role for LT signaling in lymphocyte-stromal interactions. We tested the latter hypothesis by constitutively expressing LTs on T cells. This conferred a CD44hi phenotype to T cells, indicative of an activated state, and induced fulminant thymic involution. A similar phenotype was detected in wt mice, but not in Tnfr1−/− or Ltbr−/− mice, on adoption of tgLtαβ BM. Hence this thymic pathology resulted from signaling to thymic stroma rather than from impaired thymocyte homing. We further investigated the effects of a lymphocytic choriomeningitis virus (LCMV) infection with strain WE (LCMV-WE) or strain Docile (LCMV-DOCILE) on thymic homeostasis. Infection of wt but not Tnfr1−/− mice with LCMV also led to precocious thymic involution and up-regulation of LT on CD8+ and CD4+CD8− thymocytes. This suggests that LTβR and TNFR1 signaling by T cells contributes to thymic involution both during physiological aging and in inflammatory conditions.

Materials and Methods

Mice

Animals were free of all bacterial, viral, and parasitic pathogens listed in the Federation of European Laboratory Animal Science Associations recommendations and were maintained under specific pathogen-free conditions. Housing and experimental protocols were in accordance with the Swiss Animal Protection Law and mice were held according to the law of the Veterinäramt, Kantons Zürich. LTα−/−, LTβ−/−, Tnfr1−/−, Tnfr2−/−, Ltbr−/−, and JH−/− mice were published previously.

Generation of tg Constructs

A Bluescript II KS (3.0 kb) vector with a deleted KpnI restriction site in the multiple cloning site was used. The vector was linearized with BamHI/Sall and a BamHI/Sall restriction product (~5.6 kb) of the PrP 5′/HG construct was cloned in ΔKpnI pBluePrP 5′/HG construct. Lck-promoter cassette (~3.1 kb) was isolated by digest with BamHI and NotI of the pPrP5′/HGLckSall construct and subsequent purification on a 0.7% agarose gel by gel extraction (Qiagen). The full Ltα and Ltβ open reading frames were amplified from a pLtα and pLtβ plasmids using primers that incorporate 5′ or 15 bp overhangs. Ten PCR products of each frame were amplified from a pLtα and pLtβ plasmids using primers that incorporate 5′ or 15 bp overhangs. PCR products were 610 bp for Ltα and 921 bp for Ltβ.

Primers used for LT-Prp overhangs are as follows. LTα primer forward: 5′-CGGGGTACCATGTCATTTTTAGTGGAGGCAAGCAGACTATCATCTGAGACACTGCTCGGCGTGCTCC-3′. LTβ primer forward: 5′-CGGGGTACCATGTCATTTTTAGTGGAGGCAAGCAGACTATCATCTGAGACACTGCTCGGCGTGCTCC-3′. LTβ primer reverse: 5′-CGGGGTACCATGTCATTTTTAGTGGAGGCAAGCAGACTATCATCTGAGACACTGCTCGGCGTGCTCC-3′. Ten PCR products of each cDNA were pooled, purified by gel extraction (Qiagen, according to the manufacturer’s manual), and partially digested with KpnI and MfeI. Digested PCR products were repurified and used for subcloning into a pMef/KpnI linearized ΔKpnI pBluePrP 5′/HG vector. After successful subcloning of both LT cDNAs, control digests and sequencing of Ltα and Ltβ open reading frames and flanking regions (ABI Prism Sequencer) were performed.
These ΔKpNI pBluePrP 5′HGLtα or Ltβ vectors were linearized with BamHI and NotI for the insertion of the promoter cassette. The purified lck-promoter 47 was linearized with BamHI and NotI for the insertion of the labeled probe (hybridization overnight at 65°C with a radioactively labeled probe) 32P-labeled. Residual DNA, the blot was marked, washed for 5 minutes by vacuum blotting (Pharmacia Biotech) and solutions technique. Optionally, vacuum blotting was performed at (Amersham) in 0.4M NaOH overnight by capillary blotting program.

The LT open reading frame and insertion sites of the lck-promoter were sequenced. Both vectors were linearized with a Sall, NotI digest leading to linearized PrP 5′HGlck Lta (9.0 kbp), or HG-Ick Ltβ (10 kbp). Fragments were isolated on a 0.7% agarose gel by the gel extraction method (Qiagen, according to the manufacturer’s manual) and used for male pronuclear micro-injection.

Genomic Southern Blot

Mouse tail biopsies were received in liquid nitrogen and digested in a lysis buffer containing 100 mmol/L Tris-HCl (pH 8.5), 5 mmol/L EDTA, 0.2% SDS, 20 mmol/L NaCl, 100 μg proteinase K/ml overnight at 55°C with constant rotation. Twenty μg of purified genomic DNA were digested overnight (EcoRI 100 units), and separated overnight on a 0.7% agarose gel (GIBCO BRL) gels. Genomic digest and DNA separation was checked by ethidium bromide staining. Genomic DNA was depurinated by treatment with 1M HCl for 15 minutes and 0.4M NaOH for 30 minutes and transferred to Hybond-N membranes (Amersham) in 0.4M NaOH overnight by capillary blotting technique. Optionally, vacuum blotting was performed at 50 to 55 mbar with a Vacu Gene Pump and a Gene XL blotting apparatus (Pharmacia Biotech) and solutions were used according to the manufacturer’s manual (Pharmacia). After the agarose gel was examined for residual DNA, the blot was marked, washed for 5 minutes in 2× standard saline citrate buffer, UV-crosslinked, and prehybridized according to Church and Gilbert. 48 After hybridization overnight at 65°C with a radioactively labeled probe (LTA or LTβ open reading frame), the blot was washed and subjected either to autoradiography using X-ray films (Kodak) or a PhosphorImager (Molecular Dynamics). Exposed images from the PhosphorImager were analyzed with a scanner (Aida) and signals were quantified with the Aida 2.41 imaging analysis program.

Probe Labeling

Template DNA (50 ng) was diluted in sterile water, and random primer (Nonamers or Hexamers) were added (Stratagene). After gentle mixing and incubation at 95°C for 5 minutes, short centrifugation was followed by 3 minutes incubation on ice. This was followed by addition of 5× Primer-buffer (dCTP), radioactive labeled 32P-dCTP (corresponding to the 25 μCi/Amersham Biosciences), and Klenow 5′/3′ (Prime-IT II). For primer elongation, the probe was incubated on 37°C for 75 minutes. All work with radioactivity was performed in an area designated for radioactive work.

Probe Purification

For purification a QIAquick PCR-purification kit was used, according to the manufacturer’s manual (Qiagen).

Adding the Radioactive Probe to the Membrane

Ten μl sonicated salmon sperm (Stratagene) were added to the radioactively labeled, purified probe, per ml hybridization solution (final concentration 100 μg/ml). The mixture was incubated at 95°C for 5 minutes, briefly centrifuged, and chilled on ice for 3 minutes before being added to the prehybridized membrane (2 hours at 65°C).

PCR Specific for tg(Ltα), tg(Ltα), and tg(Ltβ)/Mice

Mouse tail-lysates (2.5 μl) were used. For tg LTα the following primers were used: Forward primer (Primer 1): 5′-CGAGTATATTCAGAACTG-3′, and reverse primer (Primer 2): 5′-CAGAGAAAAACCACTGGAGAAG-3′. For tg LTβ the following primers were used: Forward primer (Primer 4): 5′-GAGTCTCTGAGAGGCTAGACAG-3′. The following PCR conditions were established on a Gene Amp PCR System 9700 PCR machine (Applied Biosystems): 95°C 60 seconds denaturation; 55°C 50 seconds annealing; and 72°C 50 seconds elongation; for 35 cycles; followed by 72°C 7 minutes shoot out, and 4°C forever cool down.

RNase Protection Assays

Mice were sacrificed with CO2. Thymi, spleens, Peyers patches, and lymph nodes were removed. Total RNA was isolated using TRIzol reagent (Life Technologies) following the manufacturer’s recommendations. Chemokine mRNA levels were determined using the Riboquant MultiGene RNase Protection Assay system (BIODN). 32P-labeled riboprobes were synthesized from a customized plasmid template set using T7 polymerase. The DNA template was digested with Dnase and total RNA (15 μg) was hybridized with the riboprobes overnight at 56°C. Single-stranded RNA species were removed by digestion with RNase A. The protected RNA species were phenol/chloroform extracted, ethanol precipitated, and electrophoresed on a 5% polyacrylamide gel. Protected chemokine probes were visualized by autoradiography of the dried gel.

Preparation of Mouse-Tail Lysates for PCR Analysis

For PCR screening of mice, 5 mm of the tail were cut and lysed in 250 μl lysis buffer containing 50 mmol/L Tris-HCl (pH 9.0), 0.5% Nonidet P-40, 0.5% Tween 20, and 0.1 mg/ml proteinase K, at 55°C with agitation overnight at 600 rpm (Thermomixer 5436, Eppendorf). Following complete digestion, proteinase K was then inactivated by...
incubation at 95°C for 10 minutes. After centrifugation (12,000 rpm/15 minutes/room temperature) the lysates were immediately used for PCR (2.5 μl/PCR reaction) or stored at −20°C.

**Histology and Immunohistochemistry**

Frozen sections from thymus (5 μm), lymph nodes, or spleen were stained with H&E. Antibodies FDC-M1 (clone 4C11; 1:50, Becton Dickinson), CD35 (8C12, PharMingen, San Diego, CA), and anti-CD45RO/ROBO220 (RA3-6B2, PharMingen) were used. CD4 for T-helper cells (clone YTS 191; 1:200) and CD8 for cytotoxic T cells (clone YTS 169; 1:50), both rat anti-mouse, were kindly provided by R. Zinkernagel. Nonlymphocytic dendritic cells-145 for dendritic cells (Biomedic AG; BMA, Switzerland, T-2013; 1:1000), peanut agglutinin for germinal-center B cells (Vector L-1070; 1:100), and F4/80 (Serotec, 1:50) for macrophages were visualized using standard methods. CK5, CK18, Ulex europeaus agglutinin-1 (UEA-1), and anti-CD45RO/B220 (RA3-6B2, Becton Dickinson), CD35 (8C12, PharMingen; fluorescein isothiocyanate [FITC]-labeled) were used. Two- and three-color FACS analyses were performed on a FACS Calibur (Becton Dickinson) as described. The following anti-mouse antibodies were used (all PharMingen): peridinin chlorophyll-a protein-labeled anti-B220, FITC-labeled anti-CD21, phycoerythrin-labeled anti-CD23, phycoerythrin-anti-CD8, FITC-anti-CD4, phycoerythrin-anti-CD11b, FITC-anti-CD8, Annexin V, and FoxP3. 7-Amino-actinomycin D (7-AAD) analysis was performed using a FACS Calibur using CELLQuest software (Becton Dickinson). Postacquisition analysis was performed using WinMDI 2.8 software and FlowJo (Institute for Neuropathology). FACS analysis and quantification of early T lineage progenitors (ETPs) was performed as described.

**Northern Blot Analysis and in Situ Hybridization**

Northern blot analysis and in situ hybridization of frozen spleen sections were performed as previously described. The following cDNA probes were used: B-lymphocyte chemotactant (nucleotides 6 to 784, GenBank U83222), secondary lymphoid organ chemokine (nucleotides 10 to 784, GenBank U83222), stromal cell-derived factor-1α (nucleotides 34 to 687), macrophage-derived chemokine (nucleotides 10 to 567), EBV-induced molecule 1 ligand CC chemokine (nucleotides 15 to 750), and complete murine GAPDH. Total RNA was prepared using TRIzol reagent (Life Technologies), and multiprobe RNase protection assay using different probe set (BD PharMingen) was performed according to manufacturer’s protocol (Oncor).

**RT-PCR**

Total thymic, splenic, or lymph nodal RNA from wt and tglLTα/β mice were isolated in TRizol reagent (Life Technologies), and reverse transcribed using the GeneAmp kit (Roche) according to the manufacturer’s instructions. Purified RNA was DNase treated according to the manufacturer’s manual (Roche). Reverse transcribed cDNA (GeneAmp kit; Roche) of total thymic, splenic, or lymph nodal RNA from wt and tgl mice was used for Taq-Man PCR. Transgene specific probes for LTα and LTβ were generated as follows: Taq-man PCR for LTα: 5’-CCAATTAGGAGCCAGCAGA-3’ and 3’ LTα Taq: 5’-TGCAACCACCTCTCAGAG-3’. Taq-man PCR for LTβ: 5’-CATCTGACACTGTCGCGGTCT-3’. Labeling of the probe was conducted as follows (reporter, 6-carboxyfluorescein; quencher, 6-carboxytetramethylrhodamin): Taq-man PCR for LTβ: 5’ Prp Taq: (the same primer as for LTα). 3’ LTβ Taq: 5’-CTGCCAGCGCCAGCAAGA-3’. Probe: 5’-CAGGCCCTGCACTCCCCCTG-3’. As control for Taq-Man PCR procedure and possible DNA contamination DNase treated RNA from wt and tgl mice that was

BM Chimeric Mice

BM cells were isolated from tibiae and femurs, and 5 to 10 × 10⁶ BM cells were injected into tail veins of 8 to 10-week-old recipients conditioned by whole-body irradiation (1100 rad) 24 hours earlier as described. Six to eight weeks after grafting, reconstitution was assessed by fluorescence-activated cell sorting (FACS) analysis of peripheral blood taken from the retro-orbital plexus of ether-anesthetized mice. Blood samples were prepared at 4°C in buffer solution (PBS containing 2% fetal calf serum and 0.2% NaN₃). Reconstitution efficiency was checked in parallel reconstitutions where wt mice received BM from mice expressing the green fluorescent protein under the chicken albumin promoter.

**FACS Analysis**

Detection of surface LTα, β on B and T lymphocytes: 1 to 5 million lymphocytes from spleen, mesenteric lymph nodes (MLN), or thymus were centrifuged at 1500 rpm for 10 minutes and washed in FACS buffer (PBS, 1 mmol/L EDTA, 0.1% Na Azide, 2% fetal calf serum) followed by incubation with 25 μl of Fc-block (anti-CD16/32 1:100 from PharMingen) in FACS buffer for 15 minutes on ice. Twenty-five μl of diluted LTβR-Fc fusion protein were added directly to the Fc-block and incubated for 30 minutes on ice. This was followed by a washing step (twice) in 200 μl of FACS buffer. The anti-human-Fc antibody (biotin: 109-0635-008; Jackson Immunoresearch) was preabsorbed for 30 minutes with 2% normal mouse and 2% normal rat serum (Sigma) to reduce the background. The biotin anti-human-Fc antibody was diluted 1:200 and after washing, a streptavidin-phycocerythrin labeled antibody (PharMingen) was added.

This procedure was followed by staining with lineage marker antibodies resuspended in normal mouse and rat serum at 1 to 2%. For T cell and B cell specific stains antibodies against CD4, CD8, CD19, and B220 (PharMingen; fluorescein isothiocyanate [FITC]-labeled) were used. Two- and three-color FACS analyses were performed on a FACS Calibur (Becton Dickinson) as described. The following anti-mouse antibodies were used (all PharMingen): peridinin chlorophyll-a protein-labeled anti-B220, FITC-labeled anti-CD21, phycoerythrin-labeled anti-CD23, phycoerythrin-anti-CD8, FITC-anti-CD4, phycoerythrin-anti-CD11b, FITC-anti-CD8, Annexin V, and FoxP3. 7-Amino-actinomycin D (7-AAD) analysis was performed using a FACS Calibur using CELLQuest software (Becton Dickinson). Postacquisition analysis was performed using WinMDI 2.8 software and FlowJo (Institute for Neuropathology). FACS analysis and quantification of early T lineage progenitors (ETPs) was performed as described.

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Cytokine Assays
Thymi were placed in 10 vol of Tris-HCl buffer (50 mmol/L, pH7.4) with NaCl (0.6 M), Triton X-100 (0.2%), and bovine serum albumin (0.5%) containing freshly dissolved protease inhibitors: benzamidine (1 mmol/L), benzethonium chloride (0.1 mmol/L), and phenylmethylsulfonyl fluoride (0.1 mmol/L). Samples were homogenized (TissueTearor; BioSpec Products, Bartlesville, OK) for 10 seconds, sonicated (Vibra Cell; Sonics & Materials, Newtown, CT) for 20 seconds at 10 mV, and centrifuged at 12,000 rpm for 20 minutes at 4°C. The supernatants were aliquoted and frozen at −80°C until the cytokine assays were performed. Cytokine protein levels in the serum and thymic homogenates were measured using a multiplexed particle-based flow cytometric cytokine assay (Vignali, 2000). Fluorokine Multi Analyte Profiling mouse kit for TNFα was purchased from R&D Systems (Wiesbaden-Nordenstadt, Germany). The procedures closely followed the manufacturer’s instructions. The analysis was conducted using a conventional flow cytometer (LSRII von Beckton Dickinson). The detection limit for TNFα was 0.4 pg/ml.

Enzyme-Linked Immunosorbent Assay for LTα3, LTα1β25, and LTα2β1
For enzyme-linked immunosorbent assay (ELISA), a Nunc Immuno Maxi-Sorp plate was coated with 0.1M Na2HPO4, pH9 at room temperature for 2 hours. After removal of 0.1M Na2HPO4, 100 μl/well of the respective 10% organ homogenate was added, and the plate was sealed and incubated overnight at 4°C. Next, the plate was washed three times with PBS/0.05% Tween, incubated in blocking buffer with 10% fetal calf serum/PBS (filtered in 0.22 μm) for 2 hours at room temperature, and subsequently washed six times with PBS/0.05% Tween.

One hundred μl of the primary antibody (MAB-748; R&D; 1:1000 diluted in blocking buffer/Tween (0.05%) were added and incubated in the sealed plate for 90 minutes at room temperature or overnight at 4°C. After subsequent washing six times with PBS/0.05% Tween, the secondary antibody (Dako #E0468) was diluted 1:2000 in blocking buffer/Tween (0.05%), 100 μl were added/well, and the plate was sealed for 1 hour at room temperature. After subsequent washing six to eight times with PBS/0.05% Tween, a strepavidin-HRP complex (Biosource SN2004; 0.95 mg/ml) was diluted 1:40,000 fold in blocking buffer/Tween (0.05%) and 100 μl were added/well. The plate was sealed and incubated at room temperature in the dark. After subsequent washing six to eight times, 100 ml of stabilized Chromogen BioSourceSBo1 was added and the plate was incubated at room temperature, wrapped in aluminum-foil, and incubated on a shaker for color development for 5 to 30 minutes. The reaction was stopped by adding 50 μl stop solution (0.5M H2SO4), color of positive wells turns yellow. The optical density of the wells was read at 405 nm on a conventional ELISA reader. For the establishment of a standard curve we used recombinant-murine “generic” LTαβ consisting of both LTα2β1 and LTα3 (kindly provided by Dr. Jeffrey Browning, Biogen Idec, Inc.) or recombinant mouse Ltα (Cat.No.749-TB/CF; R&D) (0.5 μg/100 μl to 7 ng/100 μl). For negative control homogenates derived from LTα−/− mice were used. In our hand the detection limit/background was ~15 ng/well.

Quantification of Relative Thymus Weight and Absolute Cell Number
For the analysis of relative thymus weight, body weight of the respective mice was weighted before thymi of different tg, wt, and knockout mice were isolated by microsurgery. Afterward, isolated thymi were weighted and thymic lobes were smashed to isolate connective tissue and fat from total thymic cells. Cells were diluted in PBS 1:10, 1:100, and 1:1000 and counted in a modified Neubauer chamber, as well as a CASY cell counter (Schärf Systems). For quantification of absolute thymic cells the average of at least three independent counting rounds was used.

Viruses and Peptides
The LCMV, WE strain, originally obtained from Dr. F. Lehmann-Grube (Hamburg, Germany), was propagated on L929 cells at a low multiplicity of infection and was plaqued as previously described.55 The LCMV-GP peptide KAVYNFATM (gp33) and the LCMV-NP peptide FQPNGNGFI (np396) were purchased from Neosystem (Strasbourg, France). Virus infection was performed with LCMV-WE (200 plaque-forming units) or LCMV-DOCILE (2 × 106 plaque-forming units). For densitometric analysis the Soft Imaging System AnalySIS52 (Olympus) was used.

Analysis of Insulin-Like Growth Factor-1 and Growth Hormone Serum Levels
Serum insulin-like growth factor (IGF)-I levels were measured by double-antibody IGF binding protein-blocked radioimmunoassay.56 Mouse growth hormone (GH) was measured using a radioimmunoassay (RPA 551; Amersham) with a detection range of 1.3 to 100 ng/ml.

LCMV-NP Specific ELISA
The LCMV nucleoprotein-specific ELISA has been described previously using LMCV-NP expressed by Spodoptera frugiperda 9 (Sf9) cells after infection with a recombinant baculovirus.

Footpad Swelling Reaction
The indicated amounts of LCMV-WE were injected in a volume of 50 ml in balanced salt solution into both hind footpads in experimental groups of three mice. The foot-
pad thickness was measured at the indicated time points with a spring-loaded caliper.

Construction of Tetrameric Class I-Peptide Complexes and Flow Cytometry

MHC class I (H-2D<sup>b</sup>) tetramers complexed with gp33 were produced as previously described. Briefly, H-2D<sup>b</sup> and human b2-microglobulin molecules were recombinantly expressed in E. coli (the plasmids were kindly provided by John Altman, Emory University, Atlanta, GA). Biotinylated H-2-D<sup>b</sup> peptide complexes were purified using and Äkta Explorer 10 chromatography system (Pharmacia, Sweden) and tetramerized by addition of streptavidin-phycoerythrin (Molecular Probes, Eugene, OR). At the indicated time points after immunization, animals were bled and single cell suspensions were prepared of the indicated time points after immunization, animals were bled and single cell suspensions were prepared of

Results

Homo- and Heterotrimeric LT is Increased in Transgenic Thymi

Transgenic (tg) mice expressing LT<sub>α</sub> or LT<sub>β</sub> under transcriptional control of the distal I<sub>k</sub> promoter<sup>57,58</sup> (Figure 1A) were generated by pronuclear microinjection, and screened by Southern blot analysis (Figure 1B). In addition, double tg mice expressing both LT<sub>α</sub> and LT<sub>β</sub> were generated by co-injecting both constructs. Four tg(LT<sub>α</sub>) founder mice out of 35 offspring from microinjected zy- gotes, 5 tg(LT<sub>β</sub>) out of 53, and 4 tg(LTαβ) out of 19 were identified by Southern blot and PCR analysis to harbor the respective transgenes (Figure 1B and data not shown). Two independent tg(LT<sub>α</sub>), three independent tg(LT<sub>β</sub>), and two independent tg(LTαβ) lines were obtained that expressed and passed the tg on to their progeny with a Mendelian ratio of approximately 50% (Table 1). For each construct, two lines were further bred for experimentation: tg(LT<sub>α</sub>β)856 and 857, tg(LT<sub>α</sub>)54 and 57, as well as tg(LT<sub>β</sub>)19 and 44.

RNase protection assays showed increased overall LT<sub>α</sub> and/or LT<sub>β</sub> mRNA expression in thymus, MLN, Peyer’s patches, and to a lesser extent in spleen of tg(LT<sub>α</sub>β) and LT single tg mice (Figure 1C and data not shown). Statistical analysis of three independent RPA experiments revealed a significant up-regulation of LT<sub>α</sub> and LT<sub>β</sub> mRNA in thymus (LT<sub>α</sub>: P < 0.02 and LT<sub>β</sub>: P < 0.03), MLN (LT<sub>α</sub>: P < 0.01 and LT<sub>β</sub>: P < 0.03) and spleen (LT<sub>α</sub>: P < 0.05 and LT<sub>β</sub>: P < 0.04) as evaluated for lines tg(LT<sub>α</sub>β)856 and 857. Magnetic activated cell sorting was performed to analyze LT<sub>α</sub> and LT<sub>β</sub> mRNA expression in enriched CD4<sup>+</sup> or CD8<sup>+</sup> thymic T cells of tg and wt mice. An increase in mRNA expression of LT<sub>α</sub> (16- to 32-fold) and LT<sub>β</sub> (7- to 16-fold) was detected in tgCD4<sup>+</sup> T cells. In tgCD8<sup>+</sup> T cells a less pronounced increase was detected (LT<sub>α</sub>: 5- to 16-fold; LT<sub>β</sub>: 4- to 10-fold) (see Supplemental Figure S1A at http://ajp.amijpathol.org). We also investigated the expression of additional TNF family members and cytokines in various organs. TGFβ3 mRNA expression was increased three- to fivefold in thymus and MLN of tg(LT<sub>α</sub>β) mice. Importantly, TNFα, TNFR1, and TNFR2 mRNA expression were unchanged (Figure 1C and see Supplemental Figure S1B at http://ajp.amijpathol.org). Expression of membrane bound LT<sub>α</sub> and LT<sub>β</sub> protein was analyzed by flow cytometry of B and T cells from 6-week-old tg(LT<sub>α</sub>β)856 (green), wt (dark purple), and Ltα<sup>−/−</sup> (pink) naive mice. Tg CD4<sup>+</sup> and CD8<sup>+</sup> cells expressed 5- to 40-fold more LT<sub>α</sub>β in thymus, MLN, and spleen, when compared with wt T cells (Figure 1D, upper and middle row). B220<sup>+</sup>/CD19<sup>+</sup> cells gated from MLN or spleen of wt and tg mice did not express increased LT<sub>α</sub>β in MLN, and spleen, when compared with wt T cells (Figure 1D, lower panels). In contrast to wt thymus, thymi contained B220<sup>+</sup>/CD19<sup>+</sup> DP populations, which were positive for LT<sub>α</sub>β<sub>+</sub> (Figure 1D). Therefore, tg LT<sub>α</sub>β expression was restricted to T cells with LT<sub>α</sub>β<sub>+</sub> expressing B cells in thymi.

With the currently available tools we analyzed homo- and heterotrimeric LT expression by ELISA in organ homogenates of 6 to 8-week-old mice. Thymi, MLNs, and spleens of lines tg(LT<sub>α</sub>β)856, 857 and tg(LTαβ)54 and 57 showed detectable LT expression. In contrast, we could hardly detect homo- and heterotrimeric LT expression in wt mice and lines tg(LT<sub>β</sub>)19 and 44 (Figure 1E and data not shown), and spleens and thymi of Ltα<sup>−/−</sup> lacked detectable LT expression. In the following we will mainly focus on line tg(LT<sub>α</sub>β)856. Lines tg(LT<sub>α</sub>β)857, tg(LTαβ)54 and 57 displayed identical or very similar phenotypes. tg(LT<sub>β</sub>)19 and 44 lacked an overt phenotype and behaved like wt mice in all experiments described below.

Enhanced Expression of LT on T Cells Induced Accelerated Thymic Involution

We analyzed the time course of the expression of LT and other TNF family members in wt and tg(LT<sub>α</sub>β)856 thymi by RPA followed by quantitative fluoroscopy (Figure 2A). LT<sub>α</sub> and LT<sub>β</sub> transcripts were up-regulated ~10-fold in tg(LT<sub>α</sub>β) thymi at postnatal day 1 (P1). At P7 LT<sub>α</sub> and LT<sub>β</sub> were overexpressed ~15-fold and ~3-fold, respectively, and remained increased two- to fourfold over wt mice at all time points thereafter (Figure 2A).

TG thymi were normal in size at P1 (Figure 2B, females: P < 0.059; males: P < 0.70), confirming the absence of a primary anlage defect. However, the thymic weight—both in absolute terms and as ratio to body weight—was drastically reduced at P7 and P14 (P < 0.008, Figure 2C and see Supplemental Figure S2A at http://ajp.amijpathol.org).
Figure 1. Generation and characterization of tg mice expressing T cell derived LT. A: Schematic representation of tgLTα and tgLTβ constructs used for pronuclear microinjection on the basis of the 5′HG expression cassette. Arrows 1 to 6 indicate various primers used for PCR and RT-PCR analysis (see Material and Methods). B: Genomic Southern blot analysis identified founder mice positive for tgLTα, tgLTβ or DP for tgLTαβ. LTα or LTβ, endogenous (end.) and transgenic (tg) LTα or LTβ EcoRI fragments detected by a radioactively labeled probe. kb: kilobase pairs. C: RPA showing increased LTα and LTβ mRNA expression in spleen, thymus, MLN, and Peyers patches of double and single tg mice (lines: tg(LTαβ)856 and tg(LTα)54). TNF, IL6, IFNγ, IFNβ, TGFβ1, TGFβ2, and MIF mRNA expression levels remained unchanged. (tgLTαβ) thymi mice displayed a three- to fivefold up-regulation of TGFβ3 mRNA. D: Histogram of FACS analysis for LTα and LTβ expression on T (CD4+ and CD8+) and B cells (B220+/CD19+) in spleen, thymus, and MLN. Green lines depict tg(LTαβ)856, purple fill LTα−/− and pink line wt mice. B220+/CD19+ cells positive for LTα,LTβ were detected in tg thymi. E: Analysis of LTαβ1, LTαβ2, and LTα3 in organ homogenates (thymus, spleen, MLN) of various transgenic lines, wt mice, and Ltα−/− mice by ELISA, reveals the presence of LTα3 in tgLTαβ and tgLTα lines but not in Ltα−/− mice.
Table 1. List of tg Lines and Transgene Copy Numbers

<table>
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<td>tgLtβ 6/tgLtβ 4</td>
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Lines marked in bold gave rise to tg-positive offspring, which expressed the tg and transmitted the tg in a Mendelian ratio. Number of tg copies was evaluated by phosphor-imaging and was quantified in relation to the signal that was the result of both endogenous alleles.

Pathol.org), and reduced to rudiments at P42 and at all time points thereafter until P336 (P < 0.001).

In view of the above results we evaluated LTα and β mRNA expression by Taq-Man analysis in wt thymi of older age (3, 6, and 18 months). For control, thymi of LTα/−/− and LTβ/−/− mice were used (n = 3; data not shown). We found a modest increase in LTα and LTβ mRNA expression in thymi of wt mice over time ranging from two- to eightfold (see Supplemental Figure S2B at http://ajp.amjpathol.org), which was significant at 6 and 18 months of age (6 months of age: LTα, P < 0.02; LTβ, P < 0.03; 18 months of age: LTα, P < 0.01; LTβ, P < 0.03).

Because casr, IGf-I, and GH may contribute to thymic involution,5 we assessed the serum levels of these hormones in tgLtαβ and wt littermates at the age of 6 weeks (n = 3 to 4 each). No differences were detected in serum levels of casr (tgLtαβ: 17 ± 3 nM/mL; wt: 18 ± 4 nM/mL), IGf-I (tgLtαβ: 312 ± 20 ng/mL; wt: 324 ± 31 ng/mL; n = 4 each), and GH (female tgLtαβ: 44 ± 17 ng/mL; male tgLtαβ: 49 ± 21 ng/mL; female wt: 41 ± 14 ng/mL; male wt: 46 ± 28 ng/mL; three males and three females each).

Abnormal T Cell Development in tg Thymi

Flow cytometry of tgLtαβ thymocytes derived from pooled thymi (P42) revealed a decrease of double-positive (DP) CD4+CD8+ T cells (wt: 83 to 86%; tgLtαβ: 11 to 29%). The relative prevalence of CD8+ T cells was higher in tgLtαβ (7 to 12%) than in wt mice (3 to 5%), yet their absolute number was significantly reduced (P < 0.01, Figure 2D and see Supplemental Figure S2C at http://ajp.amjpathol.org). This was confirmed by quantification of total thymocytes (see Supplemental Figure S2C at http://ajp.amjpathol.org). A relative increase was also detected in CD4+CD8+ double-negative (DN) cells (wt: 5 to 9%; tgLtαβ: 68 to 81%; Figure 2D). Absolute numbers of DN T cells were reduced in tgLtαβ (n = 4) (0.45 × 107 ± 0.1 × 107 cells/thymus) when compared with wt thymi (n = 4) (0.3 × 108 ± 0.15 × 108 cells/thymus; P = 0.001M and see Supplemental Figure S2C at http://ajp.amjpathol.org).

Tg thymi contained relatively increased CD44highCD4+ and CD44highCD8+ T cell subsets (Figure 2D, right panels, and see Supplemental Figure S2D and S2E at http://ajp.amjpathol.org). Also, DN CD25+CD44+ thymocytes were hyperproliferative, whereas DNS CD25+CD44+ thymocytes were decreased in tgLtαβ thymi (Figure 2D and see Supplemental Figure S2E at http://ajp.amjpathol.org, middle column).

To distinguish the various DN T cell subsets (DN1-DN4) from other populations in the thymus (eg, B cells, NK cells) we have excluded Lin+ thymocytes by magnetic activated cell sorting and analyzed Lin− thymocytes of tgLtαβ and wt thymi (n = 3 each) by flow cytometry analysis (Figure 2E). A significant reduction of Lin− cells within tgLtαβ thymi was observed (tgLtαβ: 0.25 × 107 ± 0.1 × 107 cells/thymus; wt: 0.45 × 108 ± 0.1 × 108 cells/thymus; P < 0.001). There was a slight decrease in the absolute number of tgLtαβ DN1 T cells (n = 5; 0.73 × 106 ± 0.11 × 106 cells/thymus) when compared to wt mice (n = 4; 0.85 × 106 ± 0.1 × 106 cells/thymus; P < 0.5), and a highly significant reduction of DN2, DN3, and DN4 T cells (P < 0.001; >2 log-fold reduction in all cases). FACS analysis for LTα,β revealed transgene expression in the DN3 and DN4 populations of tgLtαβ mice (data not shown).

Thymic early T lineage progenitors (Lin−CD25−CD44+ c-kit+ ETPs) undergo depletion in aged mice.50 We found significant depletion of ETPs already in 6-week-old tgLtαβ thymi (n = 4; tgLtαβ: 0.15 × 106 ± 0.1 × 106; n = 3; wt: 0.5 × 106 ± 0.2 × 106; n = 3; P < 0.02).

The peripheral blood of tgLtαβ mice contained fewer total leukocytes (P < 0.03), CD4+ T cells (P < 0.001), and CD8+ T cells (P < 0.002; see Supplemental Figure S3A and B at http://ajp.amjpathol.org). This was accompanied by a relative increase of CD44high lymphocytes (P < 0.02) (see Supplemental Figure S3D and C at http://ajp.amjpathol.org).

Histological examination of the tgLtαβ thymic microarchitecture revealed a conspicuous reduction of cortical areas already at P1 (Figure 3), well before the onset of discernible involution, with concomitant enlargement of the medullary compartment (Figure 3 and 4A) as assessed with antibody anti-MTS10. The latter was even more pronounced at P42 (MTS10+area mm²/20 mm² thymic tissue: P1: tgLtαβ: 6 ± 1.5, wt: 3 ± 0.4, P < 0.03; P42: tgLtαβ: 13 ± 2, wt: 3.5 ± 0.8, P < 0.001).

We further investigated whether the MTS24+ precursors of medullary and cortical stromal cells were changed in tgLtαβ mice.59 MTS24+ staining was ~10-fold increased in tgLtαβ thymi at 6 weeks of age (Figure 4B). A similar increase was detected in lines tg(Ltxβ)857, tg(Ltxβ)54, and tg(Ltxβ)57, thereby excluding any integrational artifacts (Figure 4B). The strong increase in MTS24+ staining was not detected at P14 in any tg mouse lines (data not shown) and must therefore must have developed between P14 and P42. No significant difference in the number of UEA-1+ medullary epithelial cells was detected between tgLtαβ (P < 0.6) or tgLtα (P < 0.7) and wt thymi (Figure 4C). Normal differentiation of thymic medullary epithelial cells into medullary cells in thymi of tgLtαβ mice was observed (see Supplemental
Figure 4 at http://ajp.amjpathol.org) and confocal microscopy of the cortico-medullary junction failed to reveal differences in the organization of CK5+ thymic medullary epithelial cells and CK18+ thymic cortical cells (see Supplemental Figure S5A at http://ajp.amjpathol.org).

The autoimmune regulatory element (Aire), expressed by the thymic stroma was reported to be regulated by the lymphotoxin pathway.59 We investigated the impact of T cell derived LTαβ on the Aire mRNA expression in the thymic stroma by RT-PCR analysis of tgLtαβ and wt thymi at various time points. Aire mRNA expression was similar in tgLtαβ and wt mice at 3, 6, and 9 weeks of age (see Supplemental Figure S5B at http://ajp.amjpathol.org).

LT Induced Thymic Involution in tgLtαβ Mice Depends on LTβR and TNFR1 Signaling

LTα3 and LTαβ2 signal mainly through TNFR1 and TNFR2, and through LTβR, respectively.14,26 Hence thymic involution may result from signaling through any of these receptors. We therefore crossed tgLtαβ mice to receptor-deficient mice (Figure 5). Additionally, we tested whether B cells participate in thymic involution by crossing tgLtαβ and B-cell deficient JH−/− mice.19,20,34,35,60–62 Thymic pathology was prevented in Tnfr1−/− and Ltbr−/−, but not in Tnfr2−/−, Ltα−/−, Ltβ−/−, and JH−/− mice (all 6 to 7 weeks old; n = 4 each; Figure 5A and see Supplemental Figure S6A at http://ajp.amjpathol.org), as assessed by thymus weight (tgLtαβ vs. Tnfr1 x tgLtαβ or
Increased Annexin V/7-AAD DP Cells in tg Thymi

We investigated whether accelerated thymic involution in tgLTαβ mice results from apoptosis (n = 4). Flow cytometry for Annexin V and 7-AAD revealed a strong increase of apoptotic 7-AAD/Annexin V positive cells in tg thymi (P28–42) (Figure 6A): Annexin V SP cells, indicative of apoptotic 7-AAD/Annexin V positive cells in thymi, revealed a highly significant increase in the number of positive cells, mainly in the cortical areas of the thymus (data not shown). We densitometrically analyzed the area of positivity mm²/10 mm² of various thymus sections from individual mice (n = 3) at 3 and 6 weeks of age (2 weeks: tgLTαβ: 0.78 ± 0.2 mm²/10 mm²; wt: 0.13 ± 0.04 mm²/10 mm², P < 0.005 and 6 weeks: tgLTαβ: 0.38 ± 0.3 mm²/10 mm²; wt: 0.15 ± 0.09 mm²/10 mm², P < 0.01).

Stromal TNFR1 and LTβR Signaling Induce Thymic Involution

Thymic involution may be caused by autocrine effects of LTs on T cells, or by destruction of the thymic TNFR1⁺ and LTβR⁺ stroma via tgLTαβ T cells.44,45 To address this question, we generated BM chimaeras whose LT signaling defects (either in TNFR1 or LTβR signaling) were confined to the stromal compartment.

Wt → wt, tgLTAβ → wt; wt → tgLTAβ; tgLTAβ → Tnfr1−/− and tgLTAβ → LtβR−/− chimeric mice were generated (n = 4 each; Figure 6B). These mice were either competent or deficient for TNFR1 or LTβR-mediated signaling in thymic stromal cells, whereas their hematopoietic compartment was either tgLTAβ or wt. Reconstitution experiments were controlled by transplanting BM of β-ActGFP mice63 into lethally irradiated wt mice, which led to 95 to 98% reconstitution, as assessed by flow cytometry of peripheral blood cells (n = 3; data not shown).
Figure 5. T cell triggered thymic involution is induced via TNFR1 and LTβR signaling. A: Macroscopic analysis of thymi from various knockout mice crossed to tgLt/H9251/H9252 mice (6 to 8 weeks old). Backcrossing to Tnfr1−/− and Lt/H9252 mice rescued the thymic phenotype due to Lt/H9251/H9252 overexpression on T cells. Scale bar: 2 mm. B and C: Quantitative analysis of relative thymic weight and absolute number of thymocytes from these crossing experiments confirmed this observation. D: FACS analysis of tgLt/H9251/H9252 x Lt/H9252 or tgLt/H9251/H9252 x LtBr−/− mice reveals an amount of SP CD4+ and CD8+, DP CD4+CD8+ and DN CD4+CD8+ T cell populations in thymus comparable to wt mice. A representative of three independent experiments is shown.

Figure 6. Stromal TNFR1 and LTβR signaling triggered by tg T cells is responsible for thymic involution in tgLt/H9251/H9252 mice. A: Flow cytometric analysis of total thymus cells for Annexin V and 7-AAD was performed to investigate the magnitude of apoptosis in pooled tg (n = 3) and wt thymi (n = 3). A representative of three independent experiments is shown. B: Macroscopic analysis of thymi from mice reconstituted by BM transplantation from several genotypes: wt BM into wt, tg BM into wt, Tnfr1−/− or LtBr−/− mice. Transfer of tgLt BM cells into wt mice induced thymic involution. In contrast, tgLt BM failed to induce thymic involution in Tnfr1−/− and LtBr−/− mice. C and D: Quantitative analysis of relative thymic weight and absolute number of thymocytes from the respective crossing experiments revealed prevention of the thymic, hypoplastic phenotype, particularly in Tnfr1−/− mice. E: Analysis of thymic micro-architecture confirmed normal distribution of CD4+ and CD8+ T cells in Tnfr1−/− or LtBr−/− mice crossed to tgLt/H9251/H9252 mice. Scale bars: 50 μm (left 2 columns) and 200 μm (right 2 columns).
Reconstitution of wt mice with tGLTαβ BM led to thymic rudimentalization and micro-architectural pathologies similar to those observed in tGLTαβ mice (Figure 6B and E), whereas reciprocal reconstitution (wt → tGLTαβ) did not revert macroscopic thymic involution, absolute number of thymocytes, or relative thymic weight (Figure 6B-D). In contrast, the absolute numbers of thymocytes and relative thymus weights of wt → wt chimaeras were similar to those of aged-matched (12 to 14 weeks) wt mice (Figures 6C-D and see Supplementary Figure S6B at http://ajp.amjpathol.org).

Tnfr1−/− mice reconstituted with tGLTαβ BM developed a normally sized thymus with number of thymocytes comparable to unreconstituted wt or Tnfr1−/− mice, in contrast to wt mice reconstituted with tGLTαβ BM (tGLTαβ→wt vs. tGLTαβ→Tnfr1−/−: P < 0.002) (Figure 6B-E and (see Supplementary Figure S7 at http://ajp.amjpathol.org). Therefore, stromal TNFR1-mediated signaling in non-hematopoietic thymic stromal cells induces thymic involution in the presence of tGLTαβ BM cells (Figure 6B). Similar results were observed in tGLTαβ BM→LtβR−/− chimeras. We could observe an unaltered thymus size, relative thymic weight (tGLTαβ→wt vs. tGLTαβ→LtβR−/−: P < 0.007), absolute number of thymocytes (Figure 6C-D), and undisturbed thymic micro-architecture (Figure 6E), when compared with wt mice that were reconstituted with tGLTαβ BM. These data suggest that thymic stromal TNFR1 and LtβR signaling pathways synergistically cause precocious thymic involution. Interestingly, crossings of tGLTαβ with Tnfr1−/− and LtβR−/− mice revealed that thymic involution can be partially prevented with one allele of LtβR but not with one allele of Tnfr1 in tGLTαβ mice (see Supplementary Figure S7 at http://ajp.amjpathol.org).

**T Cell-Derived LT Modulates T Cell-Dependent Immune Response in vivo**

tGLTαβ mice showed hypoplastic thymus with reduced numbers of thymic and peripheral CD4+ and CD8+ T cells. To study the functional significance of this phenotype, we investigated the immune response of tg mice on viral infection. We intravenously challenged tGLTαβ, tGLTβ, and wt mice (n = 4 each) with 200 plaque-forming units of LCMV-WE. Organs were taken 9 days post-infection and the efficiency of viral clearance was assessed by a plaque assay for LCMV-WE. In primary and secondary lymphoid organs (thymus, MLN, and spleen) the clearance of LCMV-WE was not as efficient as in wt controls (see Supplementary Figure S8A at http://ajp.amjpathol.org). Injection of LCMV-WE into footpads of mice led to markedly reduced footpad swelling in tGLTαβ mice than in tGLTβ or wt mice, indicating inefficient local CD8+ T cell response (see Supplementary Figure S8B at http://ajp.amjpathol.org). Despite this mitigated inflammatory response, the immunoglobulin class switch for antibodies specific for the LCMV nuclear protein (np396) remained unaltered (see Supplementary Figure S8C at http://ajp.amjpathol.org). Reduced activity of LCMV-specific T cells and reduced LCMV-WE specific CD8+ T cells were found by direct ex vivo killer assays in MLN, spleen, and blood (see Supplementary Figures S6D and 9 at http://ajp.amjpathol.org).

**LCMV Infection Induces Acute Thymic Involution**

Thymic involution of tGLTαβ mice with concomitant increase of CD44 expression on CD4+ and CD8+ T cells resembled a T cell activation phenotype. We therefore asked whether LCMV infection per se may induce thymic involution. Wt mice (n = 4 each) were infected with LCMV-WE or LCMV-DOCILE. Given the stronger virulence of LCMV-DOCILE when compared to LCMV-WE we anticipated a more pronounced effect in LCMV-DOCILE infected animals.

Age matched, untreated wt mice (n = 4) served as controls. Mice were sacrificed at 9 days post-infection, and thymi were investigated for size, weight, thymocyte cellularity, and micro-architecture. Macroscopically thymus size was decreased in LCMV-WE infected wt mice and even more drastically in LCMV-DOCILE infected wt mice when compared to untreated, age-matched controls (8 weeks old). (Figure 7A). Thymic involution was confirmed by quantification of the relative thymus weight and absolute cell number of thymocytes (Figure 7A and 7B). Histological analysis revealed disturbance of the thymic micro-architecture of LCMV infected wt mice with a reduction of the thymic cortex and a relative enlargement of the thymic medulla similar to what was observed in tGLTαβ mice (Figure 7C). The relative enlargement of the thymic medulla was densitometrically analyzed in LCMV-WE and LCMV-DOCILE infected animals (n = 4 each; 4 step cuts/mouse). Similar to tGLTαβ mice at P42 we observed a significant relative enlargement of the thymic medulla after infection with both, LCMV-WE and LCMV-DOCILE (MTS10− area mm2/20 mm2 thymic tissue: LCMV-WE: 8.5 ± 2.5, wt not-infected: 4.5 ± 1.5, P < 0.02; LCMV-DOCILE: 12.5 ± 3.5, wt not-infected: 4.5 ± 1.5, P < 0.003).

We noted that TNFα levels were increased in sera and thymi of LCMV-DOCILE- and LCMV-WE-infected mice (Figure 7D and data not shown). We further investigated whether blocking of TNFR1 signaling could prevent thymic involution after LCMV-DOCILE infection. Tnfr1−/− mice were infected with LCMV-DOCILE and compared to infected and uninfected wt mice (n = 4 each, female and male). Infected Tnfr1−/− mice displayed increased TNFα levels similarly or slightly higher to infected wt mice (n = 3) (Figure 7D). Infected wt mice displayed a drastic reduction of relative thymic weight and absolute thymocytes regardless of the sex of the infected mice (Figure 7E and F). Female Tnfr1−/− mice infected with LCMV-DOCILE displayed significantly less thymic involution than infected wt mice (P < 0.01), whereas rescue was marginal in male Tnfr1−/− mice (P = 0.12).

Following LCMV-WE or LCMV-DOCILE infection, Ltαββ expression was increased on the surface of CD8+ thymocytes and to lesser degree on CD4+ CD8+ T cells as investigated by flow cytometry analysis (Figure 7G and data not shown). In addition, we investigated homo- and heterotrimeric LT protein expression in thymic homogenates of LCMV-WE or LCMV-DOCILE infected animals at 9 days post-infection. We could
observe an increase in LT expression in thymic homogenates of LMCV-WE or LCMV-DOCILE infected mice but not in control C57BL/6 mice (see Supplemental Figure S10 at http://ajp.amjpathol.org).

This was accompanied by a reduction in the absolute number of DP thymic T cells of wt mice infected with LCMV-WE or LCMV-DOCILE (42 to 71%; P < 0.01) when compared to uninfected wt mice (81 to 84%). In contrast SP CD4⁺ and CD8⁺ T cells were increased in relative and absolute numbers in LCMV-WE infected mice (infected CD4⁺ T cells: 13 to 34%, P < 0.01; CD8⁺ T cells: 6 to 13%, P < 0.03; uninfected CD4⁺ T cells: 6 to 9%; CD8 T cells: 3 to 5%). Moreover, primed, tetramer (gp33-reactive) positive CD8⁺ T cells, indicative of viral specific T cells that re-
entered, were detected at 9 days post-infection in thymi of LCMV-WE-infected wt mice (see Supplemental Figure S10A and B at http://aip.ajipathol.org).

The prevalence of gp33-specific CD8+ T cells in thymus (1.5 to 3% of all thymic gated CD8+ T cells) was lower than in spleen (9 to 12% of all splenic gated CD8+ T cells) (see Supplemental Figure S10A at http://aip.ajipathol.org). In LCMV-DCiLE infected mice the amount of tetramer positive CD8+ T cells was less pronounced in spleen and thymus (data not shown). Accordingly, the absolute and relative number of splenic CD8+ T cells was only slightly, not significantly increased when compared to uninfected wt mice (data not shown). As reported earlier at that time point exhaustion of T cells in spleen and thymus of LCMV-DOCiLE infected mice could already be detected (64 and data not shown).

Discussion

Here we investigated the functional consequences of chronic overexpression of LT by T cells on thymus development and homeostasis. Multiple lines of tgLtαβ and tgLtα mice showed accelerated thymic involution, aberrant T cell development, and changes in thymic microarchitecture. None of these pathologies were observed on overexpression of LTβ alone, suggesting that LTα, whose expression is tightly controlled in T cells, is crucial to the phenotype described.65 Overexpression of T cell-derived LT did not lead to an increase of cortisol, IGF-I, or GH serum levels,66,67 indicating that the pathologies were not mediated by these hormones.

SP CD4+ and CD8+ thymic tgLtαβ T cells, as well as CD4+CD8+ T cells, were significantly reduced. Despite their relative increase within tgLtαβ thymic rudiments, the absolute numbers of DN T cells were also decreased. A breakdown of Lin- DN T cell subsets in tgLtαβ mice revealed a slight reduction of DN1 and a strong reduction of DN2, DN3, and DN4 T cell subsets.

ETPs, a subset of the DN1 T cell subset, are thought to reflect the developmental potential in the thymus and decrease over time in parallel with the progressively reduced thymic regenerative potential.53 We found that tgLtαβ thymi hosted fewer ETPs, similarly to aged wt thymi.53 Thymic atrophy of tgLtαβ went along with a strong reduction of the thymic cortex and a relative enlargement of the thymic medullary compartment, again reminiscent of age-related thymic involution.

MTS24+ cells, which are precursors of medullary and cortical stromal cells, were increased in thymi of tgLtαβ and tgLtα mice. This increase was observed at the peak of infection (P42) but not at birth, suggesting that it represents a compensatory mechanism.

Thymic involution was rescued by removal of either TNFR1 or LTβR, but not of TNFR2, LTα, LTβ, or B cells (as in JH-/- mice). This indicates that pathology is mediated by two non-redundant pathways: LTα signaling through TNFR1, and LTα,β signaling through LTβR. No up-regulation of TNFα mRNA was observed in tgLtαβ thymus and MLN.

What is the mechanism of thymic pathology? LT over-expression may conceivably lead to the cell-autonomous premature demise of thymocytes, thereby depleting the thymus of immature T cells. Alternatively, LT-expressing T cells may induce thymic involution by engaging pro-apoptotic receptors on thymic stromal cells. This question was clarified with reciprocal BM transplants. Thymic involution occurred in tgLtαβ-wt mice, but was prevented in tgLtαβ>Tnfr1-/- chimeric mice. Therefore tgLtαβ T cells primarily targeted the thymic stromal compartment, presumably via LTα. In view of phenotype of tgLtαβ x Tnfr2-/- mice, LTα-TNFαR2 signaling seems irrelevant in this context.

Membrane-bound LTα1β2 mainly signals via LTβR. Thymic involution was rescued in tgLtαβ>LTβR-/- chimeric mice, but to a somewhat lesser extent than in Tnfr1-/- recipient mice. Therefore thymic damage is executed through TNFR1 and LTβR. Finally, administration of tgLtαβ BM to wt mice resulted in thymic destruction within 6 weeks after BM transplantation, ruling out intrinsic homing defects of tgLtαβ thymocytes as the cause of thymic involution. Interestingly, hemizygous crosses revealed that thymic involution was partially prevented by removing one allele of LTβR but not one allele of TNFR1, establishing a hierarchy in the signaling events leading to thymic pathology. These results help interpreting the previously reported finding41 that T cell-specific expression of a ligand of LTβR and herpesvirus entry mediator, induced thymic involution with some similarities to the phenotypes described here.

Efficient selection and release of CD4+ and CD8+ T cells is important for both the innate and adaptive immune system, suggesting that the immune competence of tgLtαβ mice might be compromised. Indeed, we found a reduced capacity of the host immune system to cope with LCMV-WE infection.

The above results raised the possibility that the acute thymic involution induced by viral infections might be caused by LT signaling and might be preventable by interfering with TNFR1 signaling. Infection with either of two LCMV strains raised LTα1β2 expression by thymocytes and TNFα levels in sera and TNFα and homophilic LT levels in wt thymi. This coincided with thymic involution and a relative reduction of the cortical and a concomitant increase of the medullary thymic compartment. Moreover, we observed a reduction of the thymus/body weight ratio and of thymocyte numbers. Crucially, LCMV-induced thymic involution was greatly reduced in Tnfr1-/- female mice. However, this rescue was much less pronounced in Tnfr1-/- males, suggesting the contribution of gender-dependent factors most likely including steroid hormones.4–7,12

Tnfr1-/- mice were found to host an increased number of thymocytes, 42 suggesting that TNF signaling may participate both positively and negatively to the regulation of thymocyte production. Alternatively, the expression of ligands to TNFR family members by T cells appears to exert a negative-trophic effect on thymic stroma, and the resulting stromal atrophy may lead to reduced thymocyte output from the thymus.
Conditions that increase the expression of agonistic TNFR1/LTβR ligands by T cells may dramatically accelerate stromal atrophy and thymic involution. LCMV infection is an acute paradigm for such conditions. As subpopulations of T cells resident in the thymus are likely to experience activation at any time, it seems likely that the phenomena outlined above may contribute to physiological, age-dependent thymic involution.

Acknowledgments

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Supplemental Figures Legend:

Supplemental Figure S1: LT mRNA expression on CD4+ or CD8+ enriched T cells and unchanged cytokine receptor expression in lymphoid organs of tg mice. (A) LTα and LTβ expression as evaluated by Taq-Man PCR of MACS sorted/ highly enriched CD4+ or CD8+ T cells from wt or tgLtαβ thymi. Results were normalized to GAPDH and wt LTα or LTβ expression. (B) RNAse protection assay of spleen, thymus and MLN revealed no apparent changes in the expression level of the indicated cytokine receptors in 6-8 weeks old tgLtαβ mice when compared to wt mice.

Supplemental Figure S2: Accelerated thymic involution in tgLtαβ857 and tgLtαβ54 mice. (A) Macroscopic analysis of thymi derived from tg lines tg(Ltαβ)857, tg(Ltα)54 and wt mice revealed macroscopic thymic involution at P42. Scale bar: 6mm. (B) Quantitative analysis of LTα and LTβ mRNA expression in thymi of wt mice with various ages (3, 6 and 18 months old). Results were normalized to GAPDH and to LTα or LTβ expression levels in wt mice with 6 weeks of age. (C) Absolute cell numbers of DP, SP and DN thymocyte populations in wt and tgLtαβ mice. Flow cytrometry analysis shows upregulation of CD44 in CD8 or CD4 positive T cells. (D) DN T cells are CD44high and CD25low.

Supplemental Figure S3: Decreased absolute T cell numbers in thymus and peripheral blood of tgLtαβ but not tgLtβ mice. (A) Quantification of leukocytes and CD4+/CD8+ T cells in peripheral blood of wt and tgLtαβ mice. (B) Flow cytometry analysis for T cells in peripheral blood revealed a reduction of CD4+ and CD8+ T cells, (C and D) that displayed a relatively high proportion of CD44high cells in tgLtαβ but not in tgLtβ or wt mice.

Supplemental Figure S4: Normal differentiation of TECs into medullary cells in thymi of tg mice. Confocal analysis of cytokeratin 5 (CK5) and MTS10 revealed normal structure and distribution of medullary thymic epithelium cells in tgLtαβ when compared to wt thymi.
Supplemental Figure S5: Largely preserved cortico-medullary junction and unaltered Aire mRNA expression in thymi of tg mice. (A) Cortico-medullary junction was assessed by confocal microscopy for CK5⁺ thymic medullary epithelial cells and CK18⁺ thymic cortical cells indicating a preserved cortico-medullary junction in wt and tg thymi. Scale bar: 100 μm. (B) Unaltered mRNA expression of Aire in tg thymi examined at various ages when compared to wt mice. Data were normalized to GAPDH and wt Aire mRNA expression levels were set to 0.

Supplemental Figure S6: Immunohistochemical analysis of thymi from various tgLtαβ intercrossings. (A) Immunohistochemical analysis of thymi derived from LTα⁺, Tnfr2⁺, Tnfr1⁺ and JH⁺ mice and mice backcrossed to tgLtαβ mice. CD4 and CD8 as well as B220 staining for B-cells was performed. (B) Quantification of the relative thymus weight and the absolute number of thymocytes of no-reconstituted wt (12-14 weeks) as well as lethally irradiated wt mice transplanted with wt BM.

Supplemental Figure S7: Partial prevention of the hypoplastic thymic phenotype in tgLtαβ/LTβR⁺⁻ mice. To investigate the degree of TNFR1 and LTβR signalling in inducing the thymic rudiment in tgLtαβ mice we compared thymi of tgLtαβ/Tnfr1⁺⁻ and tgLtαβ/LTβR⁺⁻ knockout lines. Relative thymus weight (A) and the absolute number of thymocytes (B) was measured. The presence of only one TNFR1 wild type allele (tgLtαβ/ Tnfr1⁺⁻) was sufficient to induce (“complete”) thymic involution, comparable to tgLtαβ mice with a drastic (~90-95%) reduction in thymocytes and absolute thymic weight. However, mice backcrossed to Tnfr1⁺⁻ (tgLtαβ/Tnfr1⁻⁻) showed normal total cell number and relative thymus weight (n=6). The absence of both LTβR alleles revealed a similar pattern (n=4) as detected in tgLtαβ/Tnfr1⁻⁻ mice. Interestingly, tgLtαβ/LTβR⁺⁻ mice (n=4) displayed a significantly higher relative thymic weight (p<0.001) and absolute number of thymocytes (p<0.001) when compared to tgLtαβ/Tnfr1⁻⁻ or tgLtαβ mice.

Supplemental Figure S8: tgLtαβ mice show partially reduced anti viral responses. (A) LCMV titers in spleen, liver, kidney, MLN, thymus and blood as investigated by a lysis assay indicated that tgLtαβ mice showed a reduced clearance capacity in organs overexpressing LTαβ. (B) Injection of LCMV-WE (50 pfu) into the footpad of wt and tg
mice displayed a reduced amount of footpad swelling indicative of an inefficient local immune response. (C) Unchanged kinetics of IgM to IgG immunoglobulin class switch in tgLtαβ mice for the viral nuclear protein np396 (NP). (D) Exhaustion of T cell response against LCMV-WE (WE) antigens investigated in a T cell killer assay: Even though T cells specific against gp33 were found in tgLtαβ mice low numbers of T cells specific against np396 (NP) were found in the spleen of tgLtαβ mice, indicative of T cell exhaustion. LD= lethal dose 200 pfu.

**Supplemental Figure S9:** Reduced numbers of total CD8+ T cells and T cells specific for gp33 and np396 in LCMV-WE infected tgLtαβ mice. (A) Reduction of CD8+ T cells in spleen and blood of tgLtαβ mice. (B-D) Reduced numbers of T cells specific for np396 and gp33 were detected as stained by MHC class I (H-2Dβ) tetramers complexed with gp33 or np396.

**Supplemental Figure S10:** Quantification of tetramer positive CD8+ T cells in the thymus and the spleen after LCMV-WE infection, as well as analysis of thymic microarchitecture post infection. (A, upper panel) FACS analysis for the detection of CD8+ Tetramer+ (gp33) T cells in the thymus of LCMV-WE infected mice. (A, lower panel) FACS analysis of CD4+ and CD8+ T cells which are enriched in spleens of LCMV-WE infected mice. (B) In spleen, the relative amount of CD8+ Tetramer+ (gp33) T cells is higher when compared to thymus. (C) Aberrant microarchitecture but no evidence for follicular structures or tertiary lymphoid organs in LCMV-WE infected thymic rudiments. Scale bar: 100 μm. (D) ELISA for LTα2β1, LTα1β2 and LTα3 performed with thymic homogenates from LCMV-DOCILE and LCMV-WE infected mice (n=3). For control thymic homogenates of uninfected wt mice (MOCK) were used.