Growth Factors, Cytokines, Cell Cycle Molecules

Overexpression of Lymphotoxin in T Cells Induces Fulminant Thymic Involution

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Activated lymphocytes and lymphoid-tissue inducer cells express lymphotoxins (LTs), which are essential for the organogenesis and maintenance of lymphoreticular 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 *Ltaβ* and *Lta* 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 radioresistant 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.¹ 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.²

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.^{3–7} Age-dependent involution of the thymus parallels the age-related

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deterioration of immune responses.⁸ 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.^{7,9} In humans, thymic involution occurs between the first and fourth decades of life.¹⁰ The key factors and cellular mechanisms driving involution are poorly understood.^{11,12}

Lymphotoxins (LTs) are expressed by activated T, B, and NK cells,^{13,14} as well as by CD45⁺CD3⁺CD4⁺CXCR5⁺ lymphoid tissue inducer cells (LTi cells).¹⁵ 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.^{16–22}

Membrane-bound LT consists of heterotrimeric complexes of LT α and LT β (LT $\alpha_1\beta_2$ or LT $\alpha_2\beta_1$), whereas secreted LT (sLT α) is homotrimeric.^{23–25} LT heterotrimers signal via LT β receptor (LT β R), whereas sLT α signals mainly via the TNF receptors, TNFR1 and TNFR2.^{14,26} LT α is inducible in B and T cells, whereas LT β mRNA is constitutively expressed.^{14,23,27} LT signaling is essential to the differentiation and function of high endothelial venules^{28,29} and ectopic LT expression can induce lymphoid neogenesis at atypical sites including kidney, pancreas, and liver.^{30,31} 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.¹⁵

LT signaling deficiencies can lead to alymphoplasia, yet do not significantly prevent thymic development, thymic structure, T cell differentiation, and negative selection.^{16–22,32–35} However, LT β R signaling was found to control normal differentiation of thymic medullary epithelial cells via cross talk between thymocytes and thymic medullary epithelial cells,³⁶ and to regulate $\gamma\delta$ T cell differentiation in concert with other factors.³⁷ LT β R plays an important role in thymic negative selection of organ-specific thymocytes through thymic medullary chemokine regulation.³⁸ Thymic homeostasis and atrophy might be controlled or influenced by changes in homeostatic chemokines and cytokines.^{6,7,39,40} Administration of pro-inflammatory cytokines and steroids can drive thymic involution.^{4–7,12,39} T-cell specific expression of the ligand for herpesvirus entry mediator and LT β R induces lymphoid tissue abnormalities that affect the thymus.⁴¹ Enhanced levels of TNF after lipopolysaccharide or concanavalin A treatment may cause a similar phenotype,⁴⁰ whereas *Tnfr1*^{−/−} ablation delays age-dependent thymic involution.⁴²

Naïve T cells do not express membrane-bound or soluble LT α ,^{21,43} 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 CD44^{high} 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 *Lt β r*^{−/−} mice, on adoption of *tgLt $\alpha\beta$* BM. Hence this thymic pathology resulted from signaling to thymic stroma rather

than from impaired thymocyte homing.^{44,45} 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 LT β R 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 Veterinärämte, Kanton Zürich. *Lt α* ^{−/−}, *Lt β* ^{−/−}, *Tnfr1*^{−/−}, *Tnfr2*^{−/−}, *Lt β r*^{−/−}, and *JH*^{−/−} mice were published previously.^{19,20,34,35,46}

Generation of tg Constructs

A pBluescript II KS (3.0 kbp) 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 kbp) of the PrP 5'HG construct⁴⁷ was cloned in (Δ KpNI pBluePrP 5'HG construct). Lck-promoter cassette (~3.1 kbp) was isolated by digest with BamHI and NotI of the pPrP5'HGLckSall construct⁴⁷ and subsequent purification on a 0.7% agarose gel by gel extraction (Qiagen). The full *Lt α* and *Lt β* open reading frames were amplified from a pLT α and pLT β plasmids using primers that incorporate 50 or 15 bp overhangs with homologous sequences to the *Pmp* cDNA, incorporating the genuine anti-thymocyte globulin of the *Pmp* locus of the PrP 5'HG construct⁴⁷ and a restriction site for MfeI and KpnI. PCR products were 610 bp for *Lt α* and 921 bp for *Lt β* .

Primers used for *LT-Pmp* overhangs are as follows. LT α primer forward: 5'-CGGGGTACCAGTCCAATTTAG-GAGAGCCAAGCAGACTATCATCATGACACTGCTCGG-CCGTCTCC-3'. LT β primer forward: 5'-CGGGGTACCAGTCCAATTTAGGAGAGCCAAGCAGACTATCATCATGGGGACAGGGGACTGCAGG-3'. LT α primer reverse: 5'-CCACCTCAATTGAATCTACAGTGCAAAGGCTCC-3'. LT β primer reverse: 5'-CCACCTCAATTGAATTCACCCCACCATCACCGC-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 MfeI/KpnI linearized Δ KpNI pBluePrP 5'HG vector. After successful subcloning of both LT cDNAs, control digests and sequencing of *Lt α* and *Lt β* open reading frames and flank 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-promoter⁴⁷ was cloned into the ΔKpNI pBluePrP 5'HGLtα (13.0kbp) or ΔKpNI pBluePrP 5'HGLtβ (13.3 kbp) vectors. All restriction enzymes used were purchased from Roche and New England Biolabs.

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'HGLtα (9.0 kbp), or HG-lck 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 pronucleus 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 (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 (LTα 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 α³²P-dCTP (corresponding to the 25 μCi/Amersham Biosciences), and Klenow 5 u/μl (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(Ltαβ), 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'-CTGAGTATATTTTCAGAACTG-3', and reverse primer (*Primer 2*): 5'-CAGAGAAAACACCTGGGAG-3'. For tg LTβ the following primers were used: Forward primer (*Primer 4*): 5'-CTGAGTATATTTTCAGAACTG-3' and reverse primer (*Primer 5*): 5'-GAGTCTCTGAGAGGCTAGAG-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, Peyer's 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 Multiprobe RNase Protection Assay system (BD PharMingen). 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 μ l/PCR reaction) or stored at -20°C.

Histology and Immunohistochemistry

Frozen sections from thymus (5 μ m), 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/B220 (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.⁴⁹ 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 mouse thymus stroma-10 and 24 (MTS10 and MTS24) for confocal microscopy were used as described.⁵⁰ Terminal deoxynucleotidyl transferase-mediated dUTP staining was performed according to the manufacturer's protocol (Oncor).

BM Chimeric Mice

BM cells were isolated from tibiae and femurs, and 5 to 10 $\times$ 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.⁵¹ 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.⁵²

FACS Analysis

Detection of surface LT $\alpha_1\beta_2$ 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 μ l of Fc-block (anti-CD16/32 1:100 from PharMingen) in FACS buffer for 15 minutes on ice. Twenty-five μ l of diluted LT β R-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 μ l 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-phycoerythrin 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 FACSCalibur (Becton Dickinson) as described.⁵¹ 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 FACSCalibur 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.⁵³

Northern Blot Analysis and in Situ Hybridization

Northern blot analysis and *in situ* hybridization of frozen spleen sections were performed as previously described.⁵⁴ The following cDNA probes were used: B-lymphocyte chemoattractant (nucleotides 6 to 762, GenBank AF044196), secondary lymphoid organ chemokine (nucleotides 10 to 784, GenBank U88322), stromal cell-derived factor-1 α (nucleotides 34 to 687), macrophage-derived chemokine (nucleotides 13 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 recommendations.

RT-PCR

Total thymic, splenic, or lymph nodal RNA from wt and tgLT α/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 tg mice was used for Taq-Man PCR. Transgene specific probes for LT α and LT β were generated as follows: Taq-man PCR for LT α : 5' Prp Taq: 5'-CCAATTTAGGAGAGCCAAGCA-3' and 3' LT α Taq: 5'-TGCCAAGCACCTCAAGAG-3'. LT α specific probe: 5'-CATCATGACACTGCTCGGCGTCT-3'. Labeling of the probe was conducted as follows (reporter, 6-carboxyfluorescein; quencher, 6-carboxytetramethylrhodamin): Taq-man PCR for LT β : 5' Prp Taq: (the same primer as for LT α). 3' LT β Taq: 5'-CTGCCACAGCCAGCAAGA-3'. Probe: 5'-CAGGCCCTGCAGTCCCCGTG-3'. As control for Taq-Man PCR procedure and possible DNA contamination DNase treated RNA from wt and tg mice that was

not reverse transcribed was used. Taq-Man PCR was performed on an ABI Prism 7700 Sequence detector.

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, LTα₁β₂, 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 over night 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 streptavidin-HRP complex (Biosource SNN2004; 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 FQPGNGQFI (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 AnalySIS^D (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 by 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 LCMV-NP expressed by *Spo-doptera 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^b) tetramers complexed with gp33 were produced as previously described. Briefly, H2-D^b and human b2-microglobulin molecules were recombinantly expressed in *E. coli* (the plasmids were kindly provided by John Altman, Emory University, Atlanta, GA). Biotinylated H2-D^b 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 spleen and lymph nodes. Aliquots of 5×10^5 cells or three drops of blood were stained using 50 ml of a solution containing tetrameric class I-peptide complexes at 37°C for 10 minutes followed by staining with anti-CD8-FITC (PharMingen) at 4°C for 20 minutes. Erythrocytes in blood samples were lysed with FACS lysis solution (Becton Dickinson) and the cells were analyzed on a FACS-can flow cytometer (Becton Dickinson) after gating on viable leukocytes. For the determination of absolute cell counts, the number of total viable leukocytes was assessed in an improved Neubauer chamber. For blood, the number of total viable leukocytes was automatically determined in an Advia counter (Bayer, Germany).

Results

Homo- and Heterotrimeric LT is Increased in Transgenic Thymi

Transgenic (*tg*) mice expressing LT α or LT β under transcriptional control of the distal *lck* promoter^{57,58} (Figure 1A) were generated by pronuclear microinjection, and screened by Southern blot analysis (Figure 1B). In addition, double *tg* mice expressing both LT α and LT β were generated by co-injecting both constructs. Four *tg*(LT α) founder mice out of 35 offspring from microinjected zygotes, 5 *tg*(LT β) out of 53, and 4 *tg*(LT $\alpha\beta$) 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 α), three independent *tg*(LT β), and two independent *tg*(LT $\alpha\beta$) 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 $\alpha\beta$)856 and 857, *tg*(LT α)54 and 57, as well as *tg*(LT β)19 and 44.

RNAse protection assays showed increased overall LT α and/or LT β mRNA expression in thymus, MLN, Peyer's patches, and to a lesser extent in spleen of *tg*LT $\alpha\beta$ 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 α and LT β mRNA in thymus

(LT α : $P < 0.02$ and LT β : $P < 0.03$), MLN (LT α : $P < 0.01$ and LT β : $P < 0.03$) and spleen (LT α : $P < 0.05$ and LT β : $P < 0.04$) as evaluated for lines *tg*(LT $\alpha\beta$)856 and 857. Magnetic activated cell sorting was performed to analyze LT α and LT β mRNA expression in enriched CD4⁺ or CD8⁺ thymic T cells of *tg* and wt mice. An increase in mRNA expression of LT α (16- to 32-fold) and LT β (7- to 16-fold) was detected in *tg*CD4⁺ T cells. In *tg*CD8⁺ T cells a less pronounced increase was detected (LT α : 5- to 16-fold; LT β : 4- to 10-fold) (see Supplemental Figure S1A at <http://ajp.amjpathol.org>). We also investigated the expression of additional TNF family members and cytokines in various organs. TGF β 3 mRNA expression was increased three- to fivefold in thymus and MLN of *tg*LT $\alpha\beta$ mice. Importantly, TNF α , TNFR1, and TNFR2 mRNA expression were unchanged (Figure 1C and see Supplemental Figure S1B at <http://ajp.amjpathol.org>). Expression of membrane bound LT $\alpha_1\beta_2$ protein was analyzed by flow cytometry of B and T cells from 6-week-old *tg*(LT $\alpha\beta$)856 (green), wt (dark purple), and LT $\alpha^{-/-}$ (pink) naïve mice. *Tg* CD4⁺ and CD8⁺ cells expressed 5- to 40-fold more LT $\alpha_1\beta_2$ in thymus, MLN, and spleen, when compared with wt T cells (Figure 1D, upper and middle row). B220⁺/CD19⁺ cells gated from MLN or spleen of wt and *tg* mice did not express increased LT $\alpha_1\beta_2$ levels (Figure 1D, lower panels). In contrast to wt thymi, *tg* thymi contained B220⁺/CD19⁺ DP populations, which were positive for LT $\alpha_1\beta_2$ (Figure 1D). Therefore, *tg* LT $\alpha\beta$ expression was restricted to T cells with LT $\alpha_1\beta_2$ expressing B cells in *tg* 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 $\alpha\beta$)856, 857 and *tg*(LT α)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 β)19 and 44 (Figure 1E and data not shown), and spleens and thymi of LT $\alpha^{-/-}$ lacked detectable LT expression. In the following we will mainly focus on line *tg*(LT $\alpha\beta$)856. Lines *tg*(LT $\alpha\beta$)857, *tg*(LT α)54 and 57 displayed identical or very similar phenotypes. *tg*(LT β)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 $\alpha\beta$)856 thymi by RPA followed by quantitative fluoroscopy (Figure 2A). LT α and LT β transcripts were up-regulated ~10-fold in *tg*LT $\alpha\beta$ thymi at postnatal day 1 (P1). At P7 LT α and LT β 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.amj>—

Figure 1. Generation and characterization of *tg* mice expressing T cell derived LT. **A:** Schematic representation of *tg(Ltαβ)* and *tg(Ltα)* constructs used for pronuclear microinjection on the basis of the 5' HG expression cassette.⁵⁸ **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 *tg(Ltα)*, *tg(Ltβ)* or DP for *tg(Ltαβ)*. LTα or LTβ, endogenous (end.) and transgenic (*tg*) LTα or LTβ *EcoRI* fragments detected by a radioactively labeled probe. kbp: kilobase pairs **C:** RPA showing increased LTα and LTβ mRNA expression in spleen, thymus, MLN, and Peyer's 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. *tg(Ltαβ)* thymi mice displayed a three- to fivefold up-regulation of TGFβ3 mRNA. **D:** Histogram of FACS analysis for LTαβ2 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 LTαβ2 were detected in *tg* thymi. **E:** Analysis of LTα3, LTα1β2, and LTα2β1 in organ homogenates (thymus, spleen, MLN) of various transgenic lines, wt mice, and *Ltα^{-/-}* mice by ELISA, reveals the presence of LTα3 in *tg(Ltαβ)* and *tg(Ltα)* lines but not in *Ltα^{-/-}* mice.

Table 1. List of *tg* Lines and Transgene Copy Numbers

<i>tg</i> construct(s)	<i>tg</i> line	<i>tg</i> copy number
LckL α	<i>tg</i> (L α) 6	<i>tg</i> L α 19
	<i>tg</i> (L α) 8	<i>tg</i> L α 1
	<i>tg</i> (L α) 54	<i>tg</i> L α 1
	<i>tg</i> (L α) 57	<i>tg</i> L α 4
LckL β	<i>tg</i> (L β) 19	<i>tg</i> L β 1
	<i>tg</i> (L β) 20	<i>tg</i> L β 14
	<i>tg</i> (L β) 31	<i>tg</i> L β 7
	<i>tg</i> (L β) 44	<i>tg</i> L β 1
	<i>tg</i> (L β) 47	<i>tg</i> L β 7
LckL α and LckL β	<i>tg</i> (L $\alpha\beta$) 855	<i>tg</i> L α 4/ <i>tg</i> L β 5
	<i>tg</i> (L $\alpha\beta$) 856	<i>tg</i> L α 2/ <i>tg</i> L β 2
	<i>tg</i> (L $\alpha\beta$) 857	<i>tg</i> L α 6/ <i>tg</i> L β 4

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 L α and β mRNA expression by Taq-Man analysis in wt thymi of older age (3, 6, and 18 months). For control, thymi of L α ^{-/-} and L β ^{-/-} mice were used ($n = 3$; data not shown). We found a modest increase in L α and L β 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: L α , $P < 0.02$; L β , $P < 0.03$; 18 months of age: L α , $P < 0.01$; L β , $P < 0.03$).

Because cortisol, IGF-I, and GH may contribute to thymic involution,⁵ we assessed the serum levels of these hormones in *tg*L $\alpha\beta$ and wt littermates at the age of 6 weeks ($n = 3$ to 4 each). No differences were detected in serum levels of cortisol (*tg*L $\alpha\beta$: 17 ± 3 nmol/L; wt: 18 ± 4 nmol/L), IGF-I (*tg*L $\alpha\beta$: 312 ± 20 ng/ml; wt: 324 ± 31 ng/ml; $n = 4$ each), and GH (female *tg*L $\alpha\beta$: 44 ± 17 ng/ml; male *tg*L $\alpha\beta$: 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 *tg*L $\alpha\beta$ thymocytes derived from pooled thymi (P42) revealed a decrease of double-positive (DP) CD4⁺CD8⁺ T cells (wt: 83 to 86%; *tg*L $\alpha\beta$: 11 to 29%). The relative prevalence of CD8⁺ T cells was higher in *tg*L $\alpha\beta$ (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%; *tg*L $\alpha\beta$: 68 to 81%; Figure 2D). Absolute numbers of DN T cells were reduced in *tg*L $\alpha\beta$ ($n = 4$) ($0.45 \times 10^7 \pm 0.1 \times 10^7$ cells/thymus) when compared with wt thymi ($n = 4$) ($0.3 \times 10^8 \pm 0.15 \times 10^8$ cells/thymus; $P = 0.001$ M and see Supplemental Figure S2C at <http://ajp.amjpathol.org>).

Tg thymi contained relatively increased CD44^{high}CD4⁺ and CD44^{high}CD8⁺ 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 hyperprevalent, whereas DN CD25⁺CD44⁻ thymocytes were decreased in *tg*L $\alpha\beta$ 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 *tg*L $\alpha\beta$ and wt thymi ($n = 3$ each) by flow cytometry analysis (Figure 2E). A significant reduction of Lin⁻ cells within *tg*L $\alpha\beta$ thymi was observed (*tg*L $\alpha\beta$: $0.25 \times 10^7 \pm 0.1 \times 10^7$ cells/thymus; wt: $0.45 \times 10^8 \pm 0.1 \times 10^8$ cells/thymus; $P < 0.001$). There was a slight decrease in the absolute number of *tg*L $\alpha\beta$ DN1 T cells ($n = 3$; $0.73 \times 10^6 \pm 0.11 \times 10^6$ cells/thymus) when compared to wt mice ($n = 4$; $0.85 \times 10^6 \pm 0.1 \times 10^6$ 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 L α β ₂ revealed transgene expression in the DN3 and DN4 populations of *tg*L $\alpha\beta$ mice (data not shown).

Thymic early T lineage progenitors (Lin⁻CD25⁻CD44⁺c-kit⁺ ETPs) undergo depletion in aged mice.⁵³ We found significant depletion of ETPs already in 6-week-old *tg*L $\alpha\beta$ thymi ($n = 4$; *tg*L $\alpha\beta$: $0.15 \times 10^5 \pm 0.1 \times 10^5$; $n = 3$; wt: $0.5 \times 10^5 \pm 0.2 \times 10^5$; $n = 3$; $P < 0.02$).

The peripheral blood of *tg*L $\alpha\beta$ 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 CD44^{high} lymphocytes ($P < 0.02$) (see Supplemental Figure S3D and C at <http://ajp.amjpathol.org>).

Histological examination of the *tg*L $\alpha\beta$ thymic micro-architecture 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: *tg*L $\alpha\beta$: 6 ± 1.5 , wt: 3 ± 0.4 , $P < 0.03$; P42: *tg*L $\alpha\beta$: 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 *tg*L $\alpha\beta$ mice.⁵⁰ MTS24⁺ staining was ~ 10 -fold increased in *tg*L $\alpha\beta$ thymi at 6 weeks of age (Figure 4B). A similar increase was detected in lines *tg*(L $\alpha\beta$)857, *tg*(L α)54, and *tg*(L α)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 *tg*L $\alpha\beta$ ($P < 0.6$) or *tg*L α ($P < 0.7$) and wt thymi (Figure 4C). Normal differentiation of thymic medullary epithelial cells into medullary cells in thymi of *tg*L $\alpha\beta$ mice was observed (see Supplemental

Figure 2. Macroscopic and cellular characterization of thymic involution in *tgLtaβ* mice. **A:** RNA expression of LT and various cytokines using RPA during postnatal development (P1 to P42). One representative experiment out of three for each time point is shown. LTα and LTβ were up-regulated in *tg* thymi during early postnatal development and in adult mice. **B:** Macroscopic analysis of postnatal thymus development in *tgLtaβ* mice and negative littermates. Thymic involution of *tg* mice can be detected between P7 and 42 (scale bar: 1 mm). **C:** Time course of alterations in relative thymus weight (ie, thymus weight with respect to body weight). Each time point is the mean of at least 3 mice analyzed. Onset of thymic involution in male C57BL/6 mice is earlier than in C57BL/6 female mice.⁷ *tg* thymi start involuting between postnatal day 7 and 14. **D:** FACS analysis of *tg* and wt thymi at P42. Numbers indicate the percentage of the respective cell populations. Pooled *tgLtaβ* thymi (*n* = 3 to 4) (lower row) show a reduction in DP T cells and an inverted CD4:CD8 ratio. A representative experiment from four is shown. **E:** FACS analysis of Lin⁻ DN thymocytes of *tgLtaβ* and wt thymi (*n* = 4).

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 *tgLtaβ* and wt thymi at various time points. Aire mRNA expression was similar in *tgLtaβ* 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 *tgLtaβ* Mice Depends on LTβR and TNFR1 Signaling

LTα₃ and LTα₁β₂ 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 *tgLtaβ* mice to receptor-deficient mice (Figure 5). Additionally, we tested whether B cells participate in thymic involution by crossing *tgLtaβ* and B-cell deficient *JH*^{-/-} mice.^{19,20,34,35,60–62} Thymic pathology was prevented in *Tnfr1*^{-/-} and *Ltβr*^{-/-}, but not in *Tnfr2*^{-/-}, *Lta*^{-/-}, *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 (*tgLtaβ* vs. *Tnfr1*^{-/-} × *tgLtaβ* or

Figure 3. Analysis of thymic microenvironment in *tg* mice. Immunohistochemical analysis of *tg* and wt thymi at P1. Even though no obvious macroscopic differences were observed at P1, *tg* thymic cortex was already reduced and thymic medulla enlarged (asterisks). Scale bar: 200 μ m.

Lt β ^{-/-} × tgLt $\alpha\beta$, $P < 0.001$), absolute number of thymocytes (Figure 5B–C), and distribution of DP, DN, and single-positive (SP) T cells (Figure 5D). These data indicate that LT α_3 is expressed in *tg* mice and that it participates in the induction of the thymic phenotype.

Increased Annexin V/7-AAD DP Cells in *tg* Thymi

We investigated whether accelerated thymic involution in *tgLt $\alpha\beta$* 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 cells, increased from $1.4 \pm 0.3\%$ in wt thymi to $18 \pm 8\%$ in *tgLt $\alpha\beta$* thymi ($P < 0.002$). Annexin V/7-AAD DP cells increased from $1.3 \pm 0.2\%$ in wt to $5.5 \pm 2\%$ in *tgLt $\alpha\beta$* thymi ($P < 0.01$). Increased apoptosis of *tgLt $\alpha\beta$* thymi was also confirmed by positive terminal deoxynucleotidyl transferase-mediated dUTP staining of histological cryosections from wt and *tgLt $\alpha\beta$* thymi, revealing 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 $\text{mm}^2/10 \text{ mm}^2$ of various thymus sections from individual mice ($n = 3$) at 3 and 6 weeks of age (2 weeks: *tgLt $\alpha\beta$* : $0.78 \pm 0.2 \text{ mm}^2/10 \text{ mm}^2$; wt: $0.13 \pm 0.04 \text{ mm}^2/10 \text{ mm}^2$, $P < 0.005$ and 6 weeks: *tgLt $\alpha\beta$* : $0.38 \pm 0.3 \text{ mm}^2/10 \text{ mm}^2$; wt: $0.15 \pm 0.09 \text{ mm}^2/10 \text{ mm}^2$, $P < 0.01$).

Stromal TNFR1 and LT β R Signaling Induce Thymic Involution

Thymic involution may be caused by autocrine effects of LTs onto T cells, or by destruction of the thymic TNFR1⁺ and LT β R⁺ stroma via *tgLt $\alpha\beta$* T cells.^{44,45} To address this question, we generated BM chimaeras whose LT signal-

Figure 4. Increase in MTS24⁺ staining in *tgLt $\alpha\beta$* mice. **A:** H&E staining of wt, *tgLt $\alpha\beta$* , or *tgLt α* mice showing the relative enlargement of the thymic medullary compartment in *tg* mice when compared to wt mice. Scale bar: 400 μ m. **B:** Confocal microscopy of thymi cryosections stained for CK5, CK18, and MTS24 were performed to investigate medullary and cortical micro-architecture by confocal microscopy. No apparent differences were observed by CK5 and CK18 staining. However, an increase in MTS24⁺ staining was observed in *tg* mice. Scale bar: 50 μ m. **C:** Confocal microscopy of *tg* and wt thymi stained for UEA-1 and CK18 were performed. No differences in the distribution pattern or UEA-1/CK18 double positivity were observed between wt and *tgLt $\alpha\beta$* or *tgLt α* mice. Scale bar: 50 μ m.

ing defects (either in TNFR1 or LT β R signaling) were confined to the stromal compartment.

Wt→wt, *tgLt $\alpha\beta$* →wt; wt→*tgLt $\alpha\beta$* ; *tgLt $\alpha\beta$* →*Tnfr1^{-/-}* and *tgLt $\alpha\beta$* →*Lt β ^{-/-}* chimeric mice were generated ($n = 4$ each; Figure 6B). These mice were either competent or deficient for TNFR1 or LT β R-mediated signaling in thymic stromal cells, whereas their hematopoietic compartment was either *tgLt $\alpha\beta$* 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 *tgLtaβ* mice (6 to 8 weeks old). Backcrossing to *Tnfr1*^{-/-} and *Ltβr*^{-/-} mice rescued the thymic phenotype due to *Ltαβ* 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 *tgLtaβ* × *Tnfr1*^{-/-} or *tgLtaβ* × *Ltβr*^{-/-} 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 *tgLtaβ* 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 *Ltβr*^{-/-} mice. Transfer of *tgLtaβ* BM cells into wt mice induced thymic involution. In contrast, *tgLtaβ* BM failed to induce thymic involution in *Tnfr1*^{-/-} and *Ltβr*^{-/-} 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 *Ltβr*^{-/-} mice crossed to *tgLtaβ* mice. Scale bars: 50 μm (left 2 columns) and 200 μm (right 2 columns).

Reconstitution of wt mice with *tgLta β* BM led to thymic rudimentalization and micro-architectural pathologies similar to those observed in *tgLta β* mice (Figure 6B and E), whereas reciprocal reconstitution (wt $\rightarrow$ *tgLta β*) 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 $\rightarrow$ wt chimaeras were similar to those of aged-matched (12 to 14 weeks) wt mice (Figures 6C-D and see Supplemental Figure S6B at <http://ajp.amjpathol.org>).

Tnfr1^{-/-} mice reconstituted with *tgLta β* BM developed a normally sized thymus with number of thymocytes comparable to unreconstituted wt or *Tnfr1*^{-/-} mice, in contrast to wt mice reconstituted with *tgLta β* BM (*tgLta β* $\rightarrow$ wt vs. *tgLta β* $\rightarrow$ *Tnfr1*^{-/-}; $P < 0.002$) (Figure 6B-E and (see Supplemental 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 *tgLta β* BM cells (Figure 6B). Similar results were observed in *tgLta β* BM $\rightarrow$ *Lt β r*^{-/-} chimaeras. We could observe an unaltered thymus size, relative thymic weight (*tgLta β* $\rightarrow$ wt vs. *tgLta β* $\rightarrow$ *Lt β r*^{-/-}; $P < 0.007$), absolute number of thymocytes (Figure 6C-D), and undisturbed thymic micro-architecture (Figure 6E), when compared with wt mice that were reconstituted with *tgLta β* BM. These data suggest that thymic stromal TNFR1 and LT β R signaling pathways synergistically cause precocious thymic involution. Interestingly, crossings of *tgLta β* with *Tnfr1*^{+/-} and *Lt β r*^{+/-} mice revealed that thymic involution can be partially prevented with one allele of LT β R but not with one allele of TNFR1 in *tgLta β* mice (see Supplemental Figure S7 at <http://ajp.amjpathol.org>).

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

TgLta β mice showed hypoplastic thymi 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 *tgLta β* , *tgLt β* , 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 (thymic rudiment, MLN, and spleen) the clearance of LCMV-WE was not as efficient as in wt controls (see Supplemental Figure S8A at <http://ajp.amjpathol.org>). Injection of LCMV-WE into footpads of mice led to markedly reduced footpad swelling in *tgLta β* mice than in *tgLt β* or wt mice, indicating inefficient local CD8⁺ T cell response (see Supplemental 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 Supplemental 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 Supplemental Figures S8D and 9 at <http://ajp.amjpathol.org>).

LCMV Infection Induces Acute Thymic Involution

Thymic involution of *tgLta β* 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-WE⁶⁴ 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 reduced 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 *tgLta β* 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 *tgLta β* 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 TNF α 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 TNF α 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, LT $\alpha_1\beta_2$ expression was increased on the surface of CD8⁺ thymocytes and to lesser degree on DN 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

Figure 7. Viral infection induces thymic involution and LT expression on T cells that is partially rescued in *Tnfr1*^{-/-} mice. **A and B:** Reduction in relative thymic weight and absolute number of thymocytes in wt mice infected LCMV-WE, detected to greater extent in wt mice infected with LCMV-DOCILE at day 9 post-infection. **C:** Immunohistochemistry analysis of thymi from LCMV-DOCILE infected wt mice and controls revealed a reduction of the thymic cortex. Scale bar = 200 μm. **D:** Increased levels of TNFα in serum and thymus protein homogenate of LCMV-DOCILE infected wt and *Tnfr1*^{-/-} mice. **E and F:** Partial rescue of thymic involution in *Tnfr1*^{-/-} female mice and to a lesser degree in *Tnfr1*^{-/-} male mice, as compared with uninfected wt mice or wt mice infected with LCMV-DOCILE. **G:** FACS analysis of thymi of LCMV-WE infected and non-infected wt mice. Thymus DP cells are reduced in LCMV-WE infected wt mice, whereas SP CD4⁺ and CD8⁺ T cells are increased. Histograms gated on CD8⁺ T cells indicate a strong up-regulation of LTα₁β₂ in 2 out of 3 of thymi from LCMV-WE infected wt mice, and weak up-regulation in the third one. Interestingly, a similar but reduced effect was also detected on DN CD4⁻CD8⁻ thymocytes.

observe an increase in LT expression in thymic homogenates of LCMV-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://ajp.amjpathol.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://ajp.amjpathol.org>). In LCMV-DOCILE 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 *tgLtaβ* and *tgLta* 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 *tgLtaβ* T cells, as well as CD4⁺CD8⁺ cells, were significantly reduced. Despite their relative increase within *tgLtaβ* thymic rudiments, the absolute numbers of DN T cells were also decreased. A breakdown of Lin[−] DN T cell subsets in *tgLtaβ* 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 thymus⁵³ and decrease over time in parallel with the progressively reduced thymic regenerative potential.⁵³ We found that *tgLtaβ* thymi hosted fewer ETPs, similarly to aged wt thymi.⁵³ Thymic atrophy of *tgLtaβ* 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 *tgLtaβ* and *tgLta* mice. This increase was observed at the peak of involution (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: LTα₃ signaling through *TNFR1*, and LTα₁β₂ signaling through *LTβR*. No up-regulation of *TNFα* mRNA was observed in *tgLtaβ* thymus and MLN.

What is the mechanism of thymic pathology? LT overexpression 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 proapoptotic receptors on thymic stromal cells. This question was clarified with reciprocal BM transplants. Thymic involution occurred in *tgLtaβ*→wt mice, but was prevented in *tgLtaβ*→*Tnfr1^{−/−}* chimeric mice. Therefore *tgLtaβ* T cells primarily targeted the thymic stromal compartment, presumably via LTα₃. In view of phenotype of *tgLtaβ* × *Tnfr2^{−/−}* mice, LTα₃→TNFR2 signaling seems irrelevant in this context.

Membrane-bound LTα₁β₂ mainly signals via *LTβR*. Thymic involution was rescued in *tgLtaβ*→*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 *tgLtaβ* BM to wt mice resulted in thymic destruction within 6 weeks after BM transplantation, ruling out intrinsic homing defects of *tgLtaβ* 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 *tgLtaβ* 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 homo- as well as heterotrimeric 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.

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