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Multiple immune abnormalities in tumor necrosis factor and lymphotoxin-α double-deficient mice

Hans-Pietro Eugster, Matthias Müller, Urs Karrer[1], Bruce D. Car[2], Bruno Schnyder, Vicki M. Eng[3], Gaetane Woerly, Michel Le Hir, Franco di Padova[4], Michel Aguet[5], Rolf Zinkernagel[1], Horst Bluethmann[6] and **Bernhard Ryffel**

Swiss Federal Institute of Technology, Institute of Toxicology, Schorenstrasse 16, 8603 Schwerzenbach, Switzerland
[1]Institute for Experimental Immunology, Schmelzbergstrasse 12, 8091 Zurich, Switzerland
[4]Sandoz Ltd, Preclinical Research, 4002 Basel, Switzerland
[6]Pharmaceutical Research Gene Technology, F. Hoffmann-LaRoche Ltd, 4002 Basel, Switzerland

[2]Present address: Du Pont Merck, Research and Development, Stine-Haskell Research Center, PO Box 30, Building 320, Newark, DE 19714-0030, USA
[3]Present address: University Laboratory Animal Resources, University of Pennsylvania, Philadelphia, PA 19104-6021, USA
[5]Present address: Genentech Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080, USA

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Abstract

To investigate the roles of tumor necrosis factor (TNF) and lymphotoxin (LT)-α in the development and function of the immune system, the *Tnf* and *Lt*α genes were simultaneously inactivated in mice by homologous recombination. These mutant mice are highly susceptible to *Listeria monocytogenes* infection and resistant to endotoxic shock induced by the combined administration of D-galactosamine (D-GalN) and lipopolysaccharide (LPS). Their splenic microarchitecture is disorganized, characterized by the loss of the clearly defined marginal zone, ill defined T and B cell areas, and absence of MAdCAM-1 and reduced ICAM-1, VCAM-1 and Mac-1 expression. They are devoid of peripheral lymph nodes and Peyer's patches, and show a strong reduction of IgA^+ plasma cells in the intestinal lamina propria. The alymphoplasia is accompanied by a marked B lymphocytosis and reduced basal Ig levels. Ig depositions in the renal glomerulus and a strong up-regulation of MHC class I antigen expression on endothelial cells of different tissues are observed. The primary humoral immune response towards sheep red blood cells reveals a defective IgG isotype switch, while that against vescicular stomatitis virus is normal. The cytotoxic T cell responses are attenuated, although still effective, against vaccinia, lymphocytic choriomeningitis virus (LCMV-ARM) and LCMV-WE. In conclusion, the combined inactivation of *Tnf* and *Lt*α confirms their essential role in the normal development and function of the immune system.

Introduction

Tumor necrosis factor (TNF) is a proinflammatory cytokine involved in host defence and pathogenesis of various diseases (1,2). Soluble trimeric TNF ligands bind to TNF receptor 1 (TNFR1) and TNFR2, leading to receptor homotrimer formation (3) and consecutive triggering of various biological responses such as proliferation, cytotoxicity and apoptosis in target cells. Lymphotoxin (LT)-α, which like TNF belongs to the growing family of TNF-like ligands (4), can bind to the same receptors as TNF. Consequentely, administration of TNF and LT-α leads to similar biological responses *in vivo* and *in vitro*. In mice, TNF is mainly expressed in macrophages and T cells, whereas LT-α expression is confined to T and B cells. The redundancy in receptor binding and the overlapping expression pattern of TNF and LT-α renders a clearcut dissection of their function

Correspondence to H.-P. Eugster

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rather difficult. Therefore, the importance of both ligands to TNF receptor-mediated effects tends to be obscured in single gene knockout mice, because the loss of one ligand may be compensated by the remaining one. As both genes are very closely linked on chromosome 17, single mutations cannot be crossed together. Rather, mice deficient for both ligands have to be generated by simultaneous targeting of both genes. Prominent functions of TNF include central roles in endotoxic shock, host defence and tumor (or parasite) induced cachexia In addition TNF plays an important, yet undefined role in the pathogenesis of autoimmune diseases (5–9). LT-α deficiency has been shown to lead to aberrant development of the spleen and absence of peripheral lymph nodes (LN) (10) Recently a third ligand, LT-β, was cloned and characterized as present on the cell surface as a LT-α/2LT-β heterotrimer, able to bind to the newly described LT-β-specific receptor (11–13).

To investigate the consequences of the combined deficiency of TNF and LT-α on the immune system we generated TNF/LT-α double-deficient mice by homologous recombination in mouse embryonic stem cells (ES) We demonstrate that the TNF/LT-α double-deficient mice are viable and fertile, but show profound structural and functional defects of the immune system

Methods

Reagents

Geneticin (G418) was obtained from Calbiochem (La Jolla, CA), FIAU [1-(2-deoxy, 2-fluoro-β-D-arabinofuranosyl)-5-iodouracil] from Bristol Myers (Squibb Pharmaceutical Research Institute, Wallingford, CT), D-galactosamine (D-GalN) from Carl Roth (Karlsruhe, Germany), Concanavalin A (Con A, C5275) and lipopolysaccharide (LPS; *Escherichia coli*, serotype O111:B4) from Sigma (St Louis, MO). Biotin-conjugated isotype-specific goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM and IgA were from Southern Biotechnology Associates (Birmingham, AL), and alkaline phosphatase (AP)-conjugated streptavidin was from Jackson Immunoresearch (West Grove, PA). IgA-specific goat anti-mouse antibody (Chemicon IG108, α chain specific), rabbit anti-goat-Cy3 antibody (Jackson) and goat anti-rat-Cy3 antibody (Jackson) were purchased from Milan Analytica (LaRoche, Switzerland) Rat anti-mouse MHC class I and class II antibodies were from ATCC clones M1/42 and M5/114 respectively. The anti MAdCAM-1 antibody was a generous gift from B. Holzmann

Construction of targeting vector

A 0.8 kb *Hin*cII–*Dra*I fragment from a TNF genomic clone (14) (provided by Dr C. V. Jongeneel) was inserted in a clockwise orientation as the 3′ target into the *Hin*cII site of a modified pBLSK$^+$ plasmid lacking sites between *Cla*I and *Eag*I A *Xho*I–*Xba*I fragment carrying the *pgk-neo* cassette from pPNT (15) provided by Dr G Veres was inserted into the respective sites, placing the 3′ target beside the *neo* cassette As a 5′ target, a PCR product (5′ primer· 5′-GCGGTACCCAGTCAC-GACGTTGTAAAAC-3′; 3′ primer 5′-GGTCTAGACGGAAGA-CAGACCTTACCTC-3′) encompassing the complete 5′ region of the genomic clone (14) up to bp 1655 was digested with Asp718I and *Xba*I and subcloned into the respective sites, placing the *neo* cassette between the genomic 3′ and 5′ targets. Subcloning of a *Eco*RI–*Hin*dIII blunt-ended fragment from pPNT, carrying the thymidine kinase cassette, into the unique, blunt-ended Asp718I site resulted in the final targeting construct which was linearized by restriction with *Not*I.

Generation of TNF/LT-α double-deficient mice

Briefly, 3×10^7 GS1 ES cells (16) were transfected with 10 μg linearized targeting vector. Recombinant clones were selected in media containing G418 and FIAU (17) Mutant ES cell clones were identified by PCR analysis. Further characterization by Southern blot analysis revealed a deletion from *Lt*α exon 2 to the middle of *Tnf* exon 4 thus completely inactivating both genes (Fig. 1A). Mutant ES cell clones were injected into C57BL/6 blastocysts and implantation led in one case to chimeras and eventual germline transmission of the mutation. All mice were maintained under specific pathogen-free conditions. Permission for animal experimentation was obtained from local authorities and performed according to institutional guidelines All mice used in experiments were 6- to 8-week-old females.

Genetic analysis of the mutant locus

Genomic DNA was isolated from tail biopsies (18) Digested DNA (10 μg) was subjected to agarose gel electropheresis and subsequently transferred to nylon membranes (Hybond-N$^+$, Amersham, Zürich, Switzerland) according to standard procedures. A genomic probe (Probe A, a *Stu*I–*Bam*HI genomic fragment, see Fig. 1A) was random labeled with [α-^{32}P]dCTP (Amersham) and used for hybridization. Filters were prehybridized for 15 min at 65°C in 1% BSA, 0.5 M sodium phosphate, pH 7 2, 20 mM EDTA, 15% deionized formamide and 7% SDS. Then the denatured probe was added to the prehybridization mix and hybridization was carried out for 2.5 h. Filters were rinsed twice in 2×SSC, 1% SDS at 65°C, followed by two 30 min washes at 65°C in 2×SSC, 1% SDS and 0.2×SSC, 1% SDS respectively, and exposed to Hyperfilm-MP (Amersham).

LPS/D-GalN induced shock

Mice (6–8 weeks old) were injected i.p. with a combination of 20 mg D-GalN and varying doses of LPS (0.1, 1, 10 or 100 μg; *E. coli*, serotype O111:B4) in saline solution (0.9%). The mice were monitored for hepatic failure and lethality. Blood was drawn 6 h after treatment from mice anesthetized with methoxyflurane (Metofane; Pitman-Moore, Mundelein, IL) to analyze alanine and aspartate aminotransferases using a COBAS phara kit (F. Hoffmann-LaRoche, Basel, Switzerland) Mice demonstrating severe signs of endotoxemia were killed

Flow cytometry

Cell suspensions from thymus and spleen were prepared by gentle squeezing of organ pieces at 4°C in DMEM, 2% FCS through a wire mesh sieve. Cells were washed and resuspended in PBS, 2% FCS at 4°C and stained with directly labeled antibodies. The following antibodies were used: anti-mouse CD4–phycoerythrin (Becton Dickinson), anti-mouse CD8–FITC (Becton Dickinson), anti-mouse B220–FITC (CD45R, clone RA3-6B2; PharMingen, San Diego, CA) and anti-mouse CD3–biotin (PharMingen). As secondary reagent

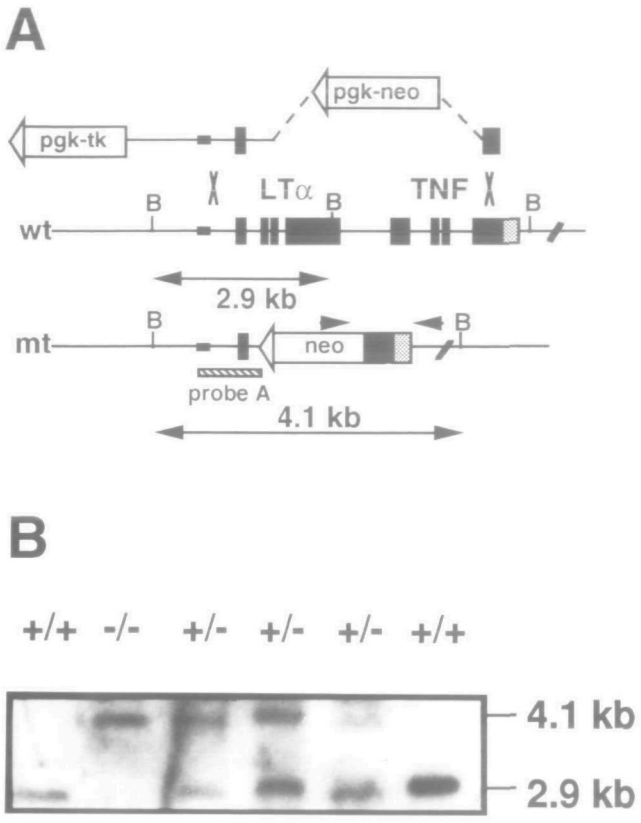

Fig. 1. Inactivation of *Tnf* and *Ltα* by homologous recombination. (A) Targeted mutation strategy showing the gene replacement vector On top, the targeting vector, in the middle the *Tnf/Ltα* locus and the predicted structure of the mutant locus at the bottom The length of the wild-type and mutated fragments detectable in the Southern blot with probe A are given. The position of the diagnostic PCR primers are indicated by arrows (B) Southern blot analysis of *Bst*EII (B) digested genomic DNA from tail biopsies of a litter from a heterozygous intercross showing wild-type, homozygous mutant and heterozygous pattern of the TNF/LT-α locus (+/+, wild-type, +/–, heterozygous, –/–, homozygous mutant).

streptavidin–Cy-Chrome (PharMingen) was used. Cells from peripheral blood were prepared by standard procedures and labeled as described above. Cells were analyzed with a FACScan flow cytometer (Becton Dickinson) using Lysys II software

Analysis of naive Ig levels and induced humoral immune response

For the determination of isotype levels, Nunc Immuno plates were coated with 5 μg/ml goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM and IgA. Bound Ig of diluted serum samples was detected with AP-coupled isotype-specific goat anti-mouse Ig antibodies. Serum Ig concentrations were calculated using mAb of the various isotypes as standards. Mice were immunized i.p. with sheep red blood cells (SRBC, 10^8 SRBC in 0.9% pyrogen free saline), and bled on days 3, 9 and 15 after immunization. Anti-SRBC serum isotype levels were determined by a sandwich ELISA Maxisorb microtiter plates (Nunc, Roskilde, Denmark) were coated with 50 μl of a solubilized extract (3 μg/ml) from SRBC prepared according to Kelly *et al.* (19) Plates were blocked with PBS/BSA 2% for 2 h at 37°C and incubated overnight with serially diluted samples. Biotinylated goat anti-mouse Ig isotype was added for 4 h Subsequently, AP-conjugated streptavidin was added for 45 min each The reaction was stopped 30 min after substrate addition with NaOH (1.5 M). Absorbance was read at 405 nm in a Titerteck Multiskan spectrophotometer (Flow Laboratories) The titer of a serum sample is expressed as the reciprocal value of the dilution showing an optical density of 0.1 over background. Neutralizing IgM and IgG titers against vesicular stomatitis virus (VSV) were determined at different time points after i v. infection with 2×10^6 p f u VSV (Indiana strain) according to established protocols (20).

Infection with Listeria monocytogenes

Mice were infected i.v with 1.2×10^3 c f u of *L monocytogenes* Animals were killed after 3 days, and listerial titers of spleen and liver were determined by plating serial dilutions of organ homogenates onto Trypticase-soy agar (21) Lethality was monitored twice daily in animals infected as described for the determination of organ titers

In vitro and in vivo anti-viral responses

For the assay of vaccinia virus (VV-WR; WR strain) specific cytotoxic T lymphocytes (CTL), mice were infected i.v. with 2×10^6 p f u. and primary *ex vivo* CTL activity was tested 6 days after infection using MC57 target cells infected with VV-WR (20). For the lymphocytic choriomeningitis virus (LCMV)-specific primary CTL response, mice were infected i.v. with 2×10^3 p f u. LCMV-ARM (Armstrong isolate) or 2×10^2 p.f.u. LCMV-WE, and primary *ex vivo* CTL activity was tested 8 days after infection using MC57 target cells infected with either LCMV-ARM or LCMV-WE For the LCMV-specific secondary CTL response, splenocytes from mice infected i.v. with LCMV-ARM (2×10^3 p.f u.) or LCMV-WE (2×10^2 p f u) were re-stimulated for 5 days *in vitro* after 8 days with LCMV-ARM or LCMV-WE infected macrophages and CTL activity was assessed as described above.

Foot pad swelling and corresponding secondary CTL were induced by injecting either 3×10^3 LCMV-ARM or 3×10^3 p f u. LCMV-WE into the hind foot pad (i.f.p.). Splenocytes were re-stimulated *in vitro* after 23 days for 5 days with LCMV-ARM- or LCMV-WE-infected macrophages and CTL activity was assessed as described above.

Histology

Sections were from freshly fixed tissue (buffered 4% formalin in PBS), paraffin embedded, cut at 5 μm and stained with hematoxylin & eosin. Tissues for immunofluorescence analysis were snap frozen in isopentane, supercooled in liquid nitrogen. Primary antibodies were applied overnight at 4°C to acetone fixed cryosections cut at 5 μm. After two washes (PBS, 1% BSA), secondary antibodies were applied for 1 h at room temperature. Slides were mounted in Shandon Immunomount and tissues were photographed in confocal mode with a Laser Scan Microscope 320 (Carl Zeiss Microscope Systems, Zürich, Switzerland) utilizing the fluorescence excitation wavelength of 543 Å. Where tissues from positive

and negative mice are presented together, identical contrast and brightness settings were employed. Negative controls (not shown) were uniformly negative

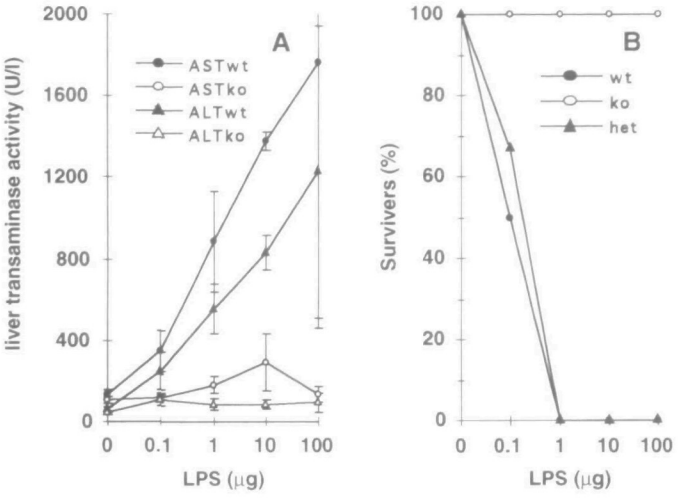

Fig. 2. (A) Liver transaminase activity in serum and (B) mortality of mutant and wild-type mice in the LPS/D-Gal sensitization model Data for liver transaminases are presented as mean units/l (± SD) Four animals were used for the two lower dose of LPS and six animals were used for the two higher doses of LPS

Results

Combined genetic inactivation of Tnf and Ltα

Simultaneous inactivation of *Tnf* and *Ltα* genes was achieved in GS1 ES cells (16) with a linearized replacement vector encompassing essential parts of both genes (Fig. 1A). The null mutation of both genes was introduced into the mouse germline by blastocyst injection using established methods. Targeting of *Tnf* and *Ltα* loci was verified in the offspring at the genomic level by Southern blot analysis from tail biopsy derived DNA (Fig 1B). In contrast to control mice, neither transcripts nor bioactive TNF could be demonstrated from LPS and Con A stimulated BMDM and splenocytes respectively (not shown). The generated TNF/LT-α double-deficient mice represent the first mice devoid of TNF- and LT-α-dependent signaling. The macroscopic phenotype of TNF/LT-α double-deficient mice consists of a slightly reduced birth weight and reduced body weight in adult mice and absence of LN Homozygous mutant mice are viable and have normal litter size

Protection from LPS/D-GalN-induced hepatic failure and endotoxic shock

TNF has been shown to be a central mediator of endotoxemia and neutralizing antibodies to TNF could protect mice from endotoxic shock (22) We therefore assessed our TNF/LT-α double-deficient mice in the LPS/D-GalN model While wild-type mice succumbed to cardiovascular shock and acute hepatic failure at 1 μg LPS, in the presence of 20 mg D-GalN,

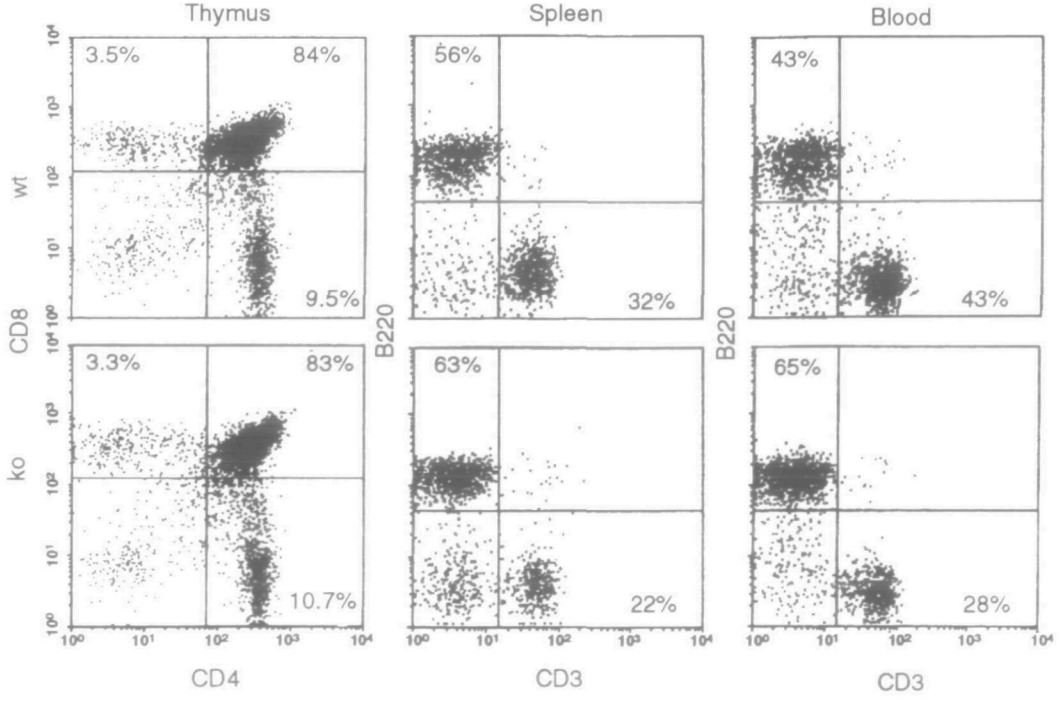

Fig. 3. Cytofluorometric analysis of thymus, spleen and blood cells. (A) Thymocytes, (B) T and B lymphocytes in spleen, and (C) peripheral blood

TNF/LT-α double-deficient mice survived a 100 times higher dose of LPS (Fig. 2). No toxicity was observed after administration of D-GalN alone. These results confirm a key role of TNF in this endotoxic shock model.

Abnormalities of secondary lymphatic organs and lymphocytosis

It has been shown that administration of TNF antibodies caused atrophy of thymus and LN (23). We therefore investi-

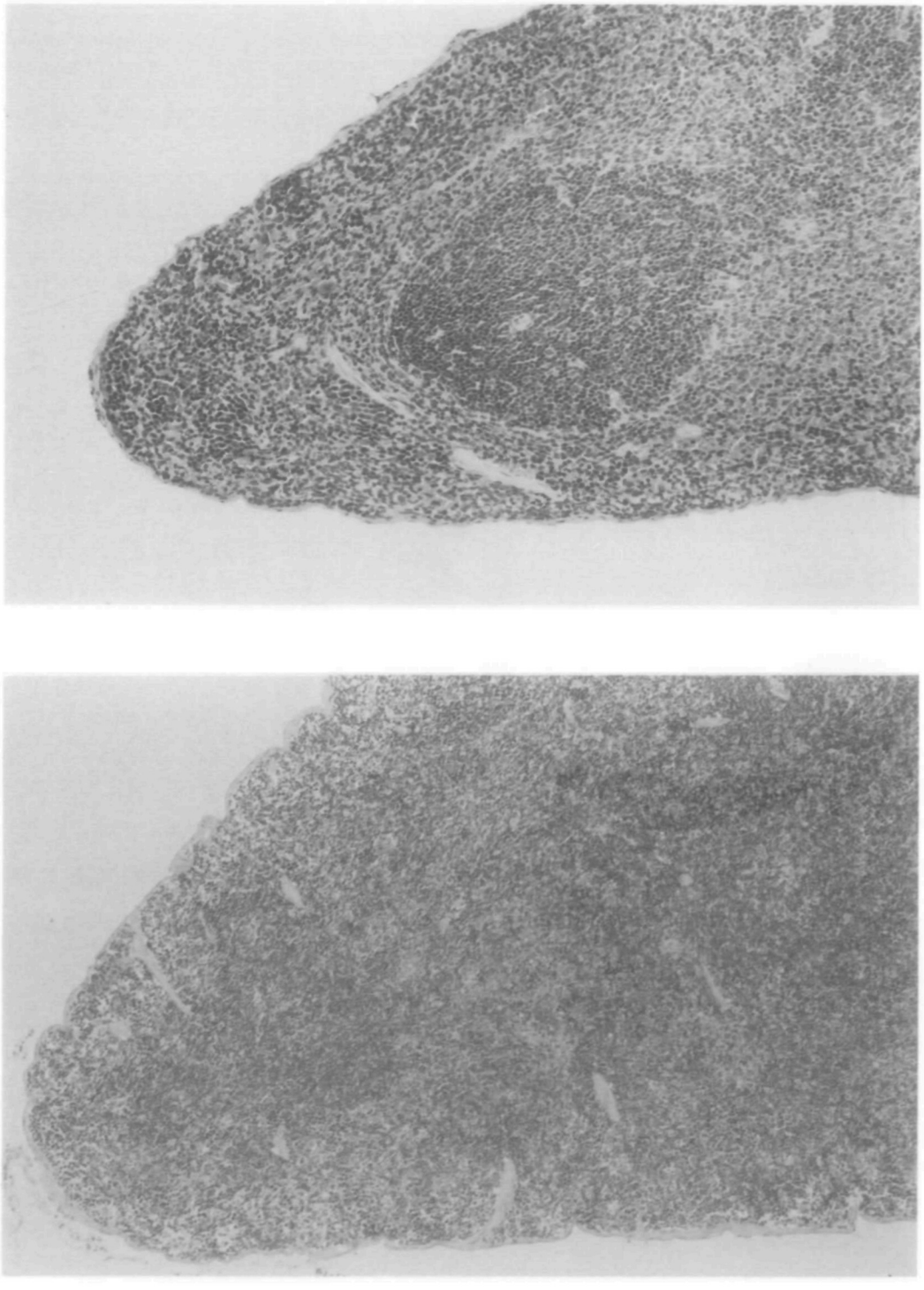

Fig. 4. Altered microarchitecture in mutant spleen. Hematoxylin & eosin stains of (A) wild-type (×200) and (B) mutant (×100) spleen showing the absence of a clearly defined marginal zone in the mutant spleen.

gated whether these alterations also occurred in our TNF/LT-α-deficient mice. The size and microscopic structure of mutant thymi, however, were normal. In addition the percentage of single $CD4^+$, $CD8^+$ and $CD4^+CD8^+$ double-positive thymocytes was not altered in mutant mice (Fig 3). On the other hand, mutant mice were completely devoid of LN and PP. Microscopic analysis of the tissues at the sites of LN revealed the presence of lymphatic vessels, but no anlage of lymphoid organs were detectable (not shown). Hence, the only peripheral lymphoid organ present in mutant mice is the spleen, which has a normal weight, size and cellularity, but exhibits a slightly altered lymphocyte composition (Fig 3) and an altered microarchitecture (Fig. 4). The splenic microarchitecture is characterized by a loss of clearly defined T cell zones and the absence of MAdCAM-1 expression in the ill-defined marginal zone (Fig 5). The expression of other adhesion molecules such as ICAM-1, VCAM-1 and Mac-1 is remarkably reduced in the marginal zone and red pulp area of mutant mice (not shown). We further asked whether the leukocyte counts in peripheral circulation were increased in this context. The hemogram showed indeed a 4-fold increase of total leukocyte counts (Table 1). Cytofluorometric analysis revealed

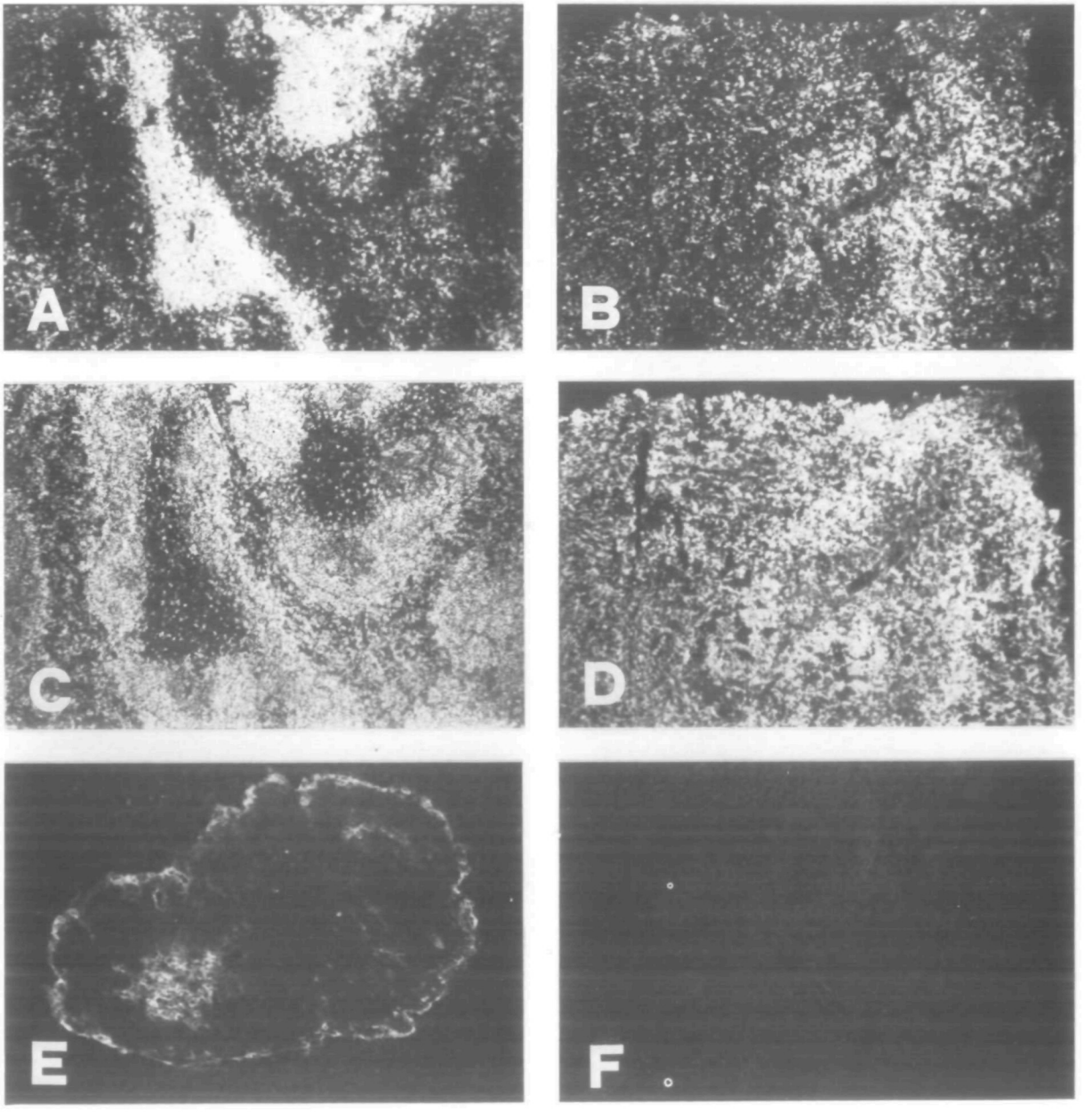

Fig. 5. Immunofluorescent analysis of spleen. Distribution of $CD3^+$ T cells (A) in wild-type and (B) mutant spleen, showing the dissolved T cell zone in mutant spleen. Distribution of $B220^+$ cells (C) in wild-type and (D) mutant spleen showing the dissolved B cell zone in the mutant spleen. MAdCAM-1 expression in (E) wild-type and (F) mutant spleen respectively. (A) and (C) as well as (B) and (D) show areas of consecutive sections (×320).

Table 1. Hematology of TNF/LT-α-deficient and control mice

Genotype	PLT ($\times 10^6$)	RBC ($\times 10^9$)	WBC ($\times 10^6$)	PMN (%)	Lympho (%)	Monos (%)	Eos (%)
(-/-)	510±41	8.35±0.7	37 1±7 1	4.2±2 1	93.8±3.5	1.2	0.6
(+/+)	484±81	8 69±0 2	9.7±2.5	8.4±5.2	88.0±6 5	0.6	3

Values are given as mean±SD (n = 5).

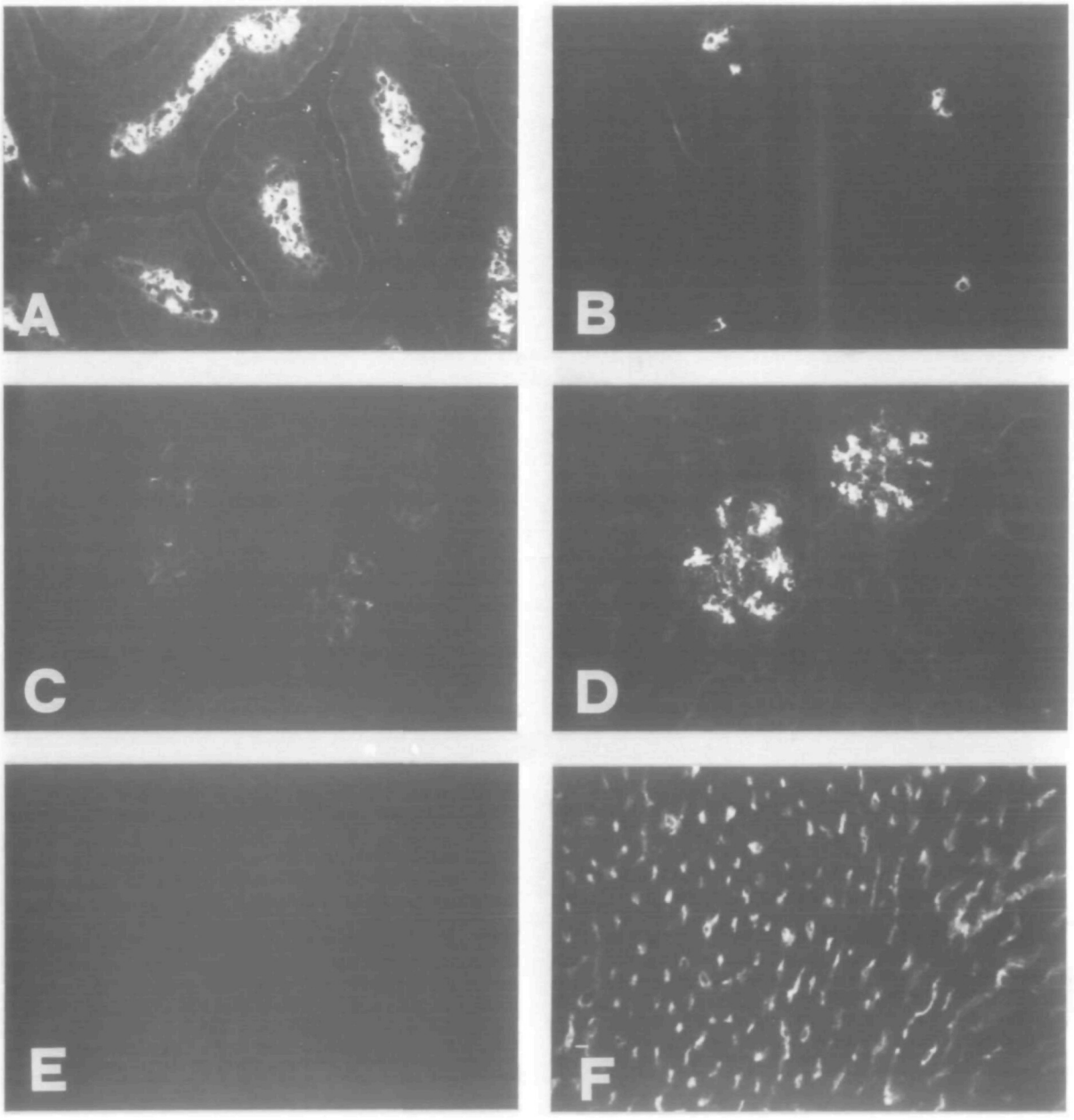

Fig. 6. Decreased incidence of intestinal IgA-specific plasma cells, renal glomerular Ig deposits and aberrant MHC class I expression in mutant mice. (A) Ileum from a wild-type mouse showing intensely IgA^+ plasma cells in the lamina propria. The epithelial cells are weakly IgA^+ (×450) (B) Duodenum of a mutant mouse with dramatic reduction of IgA^+ plasma cells and no fluorescence in the epithelium (×450). (C) Renal cortex of a wild-type mouse with background IgG2a-specific glomerular deposits (×400). (D) Renal cortex of a mutant mouse showing increased amount of IgG2a-specific glomerular deposits (×400). (E) MHC class I expression in myocard of wild-type mice is below detection level. (F) Marked MHC class I up-regulation is apparent in intermyocardial fiber capillary endothelia (×400) in mutant myocard.

Table 2. Basal isotype levels of TNF/LT-α-deficient and control mice

Genotype	IgM	IgG1	IgG2a	IgG2b	IgG3	IgA
(+/+)	370±46	144±39	95±39	382±114	66±23	63±13
(-/-)	253±17	28±5.6	27±5 4	28±10	13±2 8	1 4±0 4

Values are given as mean±SD (n = 5)

65 ± 4% $B220^+$ B cells in mutant mice compared with 44 ± 2% in wild-type mice and a relative decrease of $CD3^+$ T cells from 45 ± 3% in wild-type to 25 ± 3% in mutant mice (Fig. 3). Considering the absolute lymphocyte counts, mutant mice have significantly increased peripheral lymphocyte counts with a 6-fold increase of B cells and a 2-fold increase in T cells. No evidence of increased B lymphopoiesis could be found in the bone marrow (data not shown).

PP represent the adequate environment for differentiation of precursors of IgA^+ plasma cells (24) Immunohistochemical analysis revealed dramatically reduced numbers of IgA^+ plasma cells in ileum (Fig 6A and B) and duodenum (not shown) of the mutants Collectively, TNF/LT-α-deficient mice show an altered splenic architecture and a complete loss of other peripheral lymphoid organs with a significant lymphocytosis

Ig deposits in renal mesangium and MHC class I up-regulation in different organs

Since TNF has been reported to play an important role in autoimmunity (25), we investigated MHC expression in different organs and the kidney for Ig deposits. Immunohistological analysis of the kidney of 3-month-old mutant mice revealed granular mesangial Ig deposits (Fig. 6C and D) of the IgM, IgG1, IgG2a and IgA isotypes in the absence of complement and any signs of active renal disease. Moreover, TNF/LT-α-deficient mice show an unexpected distinct up-regulation of MHC class I expression on capillary endothelial cells in the myocardium (Fig. 6E and F), lung, thymic medulla and kidney (not shown) compared with control animals. MHC class II expression was normal. Despite this alteration in class I expression and glomerular Ig deposits, TNF/LT-α mice show no evidence of an autoimmune disorder.

Ig levels and antibody response

We then asked to what extent the basal Ig synthesis and the humoral immune response was affected in the context of alymphoplasia, disorganized spleen and B lymphocytosis. We found a distinct reduction of IgM and all IgG isotypes; and in accordance with the strong reduction of mucosal IgA producing plasma cells, very low IgA levels in the serum (Table 2) The antibody response was measured after immunization with VSV or SRBC. Neutralizing Ig titers against VSV were determined at different time points, on day 4 for the T-cell-independent IgM response and on days 8 and 12 for the strictly T-cell-dependent IgG response. Surprisingly, no differences between mutant and wild-type neutralizing IgM and IgG titers against VSV were detectable and the antibody response correlated with the clearance of VSV (not shown). However, the SRBC-specific antibody response was markedly altered. Mutant mice were unable to mount a primary IgG response against SRBC (Table 3, only IgG1 titers are shown) Thus, despite the observed B cell lymphocytosis, basal Ig levels are reduced and the response to a T-cell-dependent antigen is largely abrogated.

Table 3. SRBC-specific immune response

Day	Genotype	IgM	IgG1
3	(+/+)	8640±1880	113±22
	(-/-)	32,940±9650	176±35
6	(+/+)	111,780±30,280	3940±1170
	(-/-)	35,400±9540	232±90
15	(+/+)	9900±1800	66,700±21,890
	(-/-)	38,700±10,950	1425±880

Values are given as mean±SEM

Anti-viral activity

TNF has been shown to exert variable effects on anti-viral activity *in vivo* and *in vitro* (26–30). Wild-type and mutant mice were therefore tested for their CTL responses against VV-WR, LCMV-ARM and LCMV-WE The primary *in vivo* CTL response from mutant mice infected i.v. with VV was slightly reduced (not shown) but strongly reduced in the case of LCMV-ARM and LCMV-WE (Fig. 7A and B). Nevertheless, secondary CTL responses (day 8) against LCMV-ARM and LCMV-WE are present, although still reduced compared with control mice (Fig. 7C and D). These CTL present were able to clear the virus from spleen and liver by day 20 (not shown) If mice were infected into the foot pad (3000 p f.u.), secondary CTL were present with LCMV-ARM, but not with LCMV-WE (Fig. 7E and F), demonstrating the lack of LCMV-WE-specific memory CTL. In addition, footpad swelling was absent after infection with both viral strains in contrast to control mice (not shown).

Anti-listerial activity

TNF plays an important role in the defence against intracellular bacteria such as *L monocytogenes* and *Mycobacterium bovis* (17,31,32). Therefore, mutant and wild-type mice were infected with low titers of *L monocytogenes*. In contrast to wild-type mice, mutant mice could not control this dose of *L. monocytogenes*, and eventually die from listeriosis characterized by strongly increased titers of *L. monocytogenes* in liver and spleen (Fig. 8A) and large necrotic lesions with boundaries of heavily infected hepatocytes (Fig. 8B).

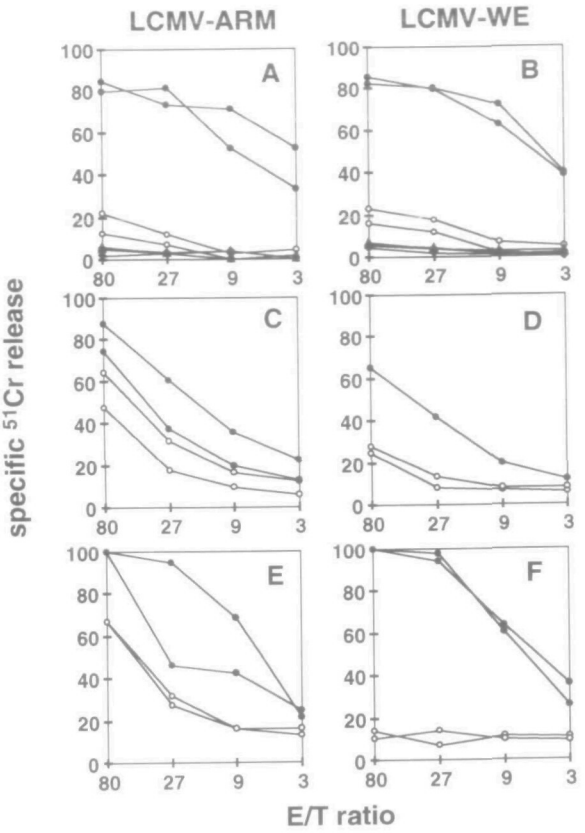

Fig. 7. Primary and secondary CTL responses against LCMV-ARM and LCMV-WE Filled symbols represent wild-type data, open symbols represent data from mutant mice. Each curve represent results from one mouse. All data points are mean values of duplicate assays. Primary CTL responses (day 8) after i.v. priming with (A) 2×10^3 p f.u LCMV-ARM and (B) 2×10^2 p.f.u LCMV-WE Secondary CTL responses (day 8) after i v priming with (C) 2×10^3 p.f.u LCMV-ARM and (D) 2×10^2 p f.u. LCMV-WE. Secondary CTL responses after i f.p. injection of (E) 3×10^3 LCMV-ARM and (F) 3×10^3 p.f u LCMV-WE Spontaneous lysis of all used target cells in absence of effector cells was <15%. Unspecific lysis of uninfected target cells was <20% in all assays.

Discussion

Simultaneous deletion of the closely linked and homologous genes coding for TNF and LT-α resulted in viable and fertile homozygous mutant animals which allowed us to investigate the specific phenotypic alterations in the immune system caused by the lack of TNF and LT-α.

Investigations using TNF neutralizing approaches revealed a central role for TNF as a mediator of endotoxic shock (22,33–35). Genetic inactivation of TNFR1 (17,31), but not TNFR2 (36), provides resistance towards LPS-induced endotoxic shock after sensitization with D-GalN. The fact that T cells represent the main source of LT-α in adult mice and that SCID mice, devoid of T and B cells, are responsive in the LPS/D-GalN model of shock (37), precludes an important role of LT-α in the pathogenesis of LPS-induced endotoxic shock. Our data, which show complete protection of TNF/LT-α-deficient mice from LPS/D-GalN-induced hepatic failure and endotoxic shock (Fig. 2), confirm the central role of TNF in this endotoxic shock model.

TNF and LT-α are expressed in the adult thymus (38) and TNF neutralization during gestation and the first 19 days of life lead to a marked atrophy of thymus, spleen and LN, and lymphopenia (23). *In vitro*, TNF induces thymocyte apoptosis (39) and either ligand mediates proliferation of immature and mature T cells *in vitro* (40). Together, these findings suggest an important role of TNF and/or LT-α in T cell maturation and the development of primary and secondary lymphatic organs. In our TNF/LT-α-deficient mice, however, thymus morphology as well as thymocyte development was normal, as it was reported for TNFR1- and TNFR2-deficient mice (31,36). However, we see alterations of spleen morphology and a complete absence of LN and PP. As TNFR1- and TNFR2-deficient mice do not exhibit these abnormalities, signaling through the newly discovered LT-β receptor is likely to be responsible for morphogenesis of LN. LT-α-deficient mice revealed similar alterations in spleen microarchitecture and absence of LN (10). Together, this defines the LT-α/LT-β-receptor interaction as the cognate ligand–receptor signaling necessary for proper spleen and LN development. Systemic absence of LN and PP and presence of ill defined splenic follicles have also been reported for the *aly* mouse (41) These overlapping phenotypic traits suggest that the *aly* gene product is involved in the LT-α/LT-β-receptor signaling pathway.

The distinct lymphocytosis observed in TNF/LT-α-deficient mice is also found in the LT-α-deficient mice (10), but not in *aly* mice (41). Therefore the lack of LN and PP cannot be invoked as the explanation for this observation, as all three mutants are devoid of these peripheral lymphatic organs Adhesion molecules play a critical role in leukocyte emigration (42), and it has been shown that TNF can induce MAdCAM-1, ICAM-1 and VCAM-1 (43). The complete absence of splenic MAdCAM-1 expression combined with the marked reduction of ICAM-1, VCAM-1 and Mac-1 expression in the marginal zone and splenic follicles of mutant mice might be responsible for the reduced margination of lymphocytes and as a consequence for the observed lymphocytosis. The fact that also ICAM-1 knockout mice present a similar leukocytosis (44) supports a causal relationship between leukocytosis and decreased adhesion molecule expression in the TNF/LT-α-deficient mouse.

A mucosal IgA deficiency was also seen in IL-6-deficient and in *aly* mice (41,45) In *aly* and the TNF/LT-α-deficient mice, this deficiency is likely due to the absence of PP and might lead to decreased mucosal immunity as shown in IL-6-deficient mice (46). The coincidence of IgA deficiency and glomerular Ig deposits of IgM, IgG and IgA isotypes is intriguing, since in man, IgA deficiency is associated with the presence of auto-antibodies, including anti-IgA antibodies (47). IgA deficiency is the most common form of immunodeficiency in man, and its incidence is increased in patients with allergies, autoimmune and gastrointestinal tract diseases (47).

The aberrant expression of MHC class I molecules on the endothelial cells of different organs suggests a negative regulatory function of basal TNF or LT-α levels for MHC class I expression. TNF has been shown to induce MHC class I

expression *in vitro* via an NFκB-like activity (48,-49), and TNF and LT-α transgenic mice showed increased expression of MHC class I on islet cells (8). In contrast, TNF induced c-Jun in 3T3-L1 pre-adipocytes and osteoblastic MC3T3-E1 cells (50,51), and c-Jun has been shown to inhibit class I expression in murine L cells (52). These data illustrate the different action of TNF and/or LT-α on the expression of class I genes which are mediated, on the one hand, by an NFκB-like activity and, on the other hand, by the c-Jun/AP-1 transcription factor. A possible explanation for the aberrant class I expression might be that the lack of basal TNF or LT-α levels leads to a down-regulation of c-Jun/AP-1 and to the loss of its negative regulatory effect on class I transcription resulting in increased basal class I expression. Whether this aberrant MHC class I expression is reversible by reintroduction of a TNF or LT-α transgene and if the aberrant class I expression might promote

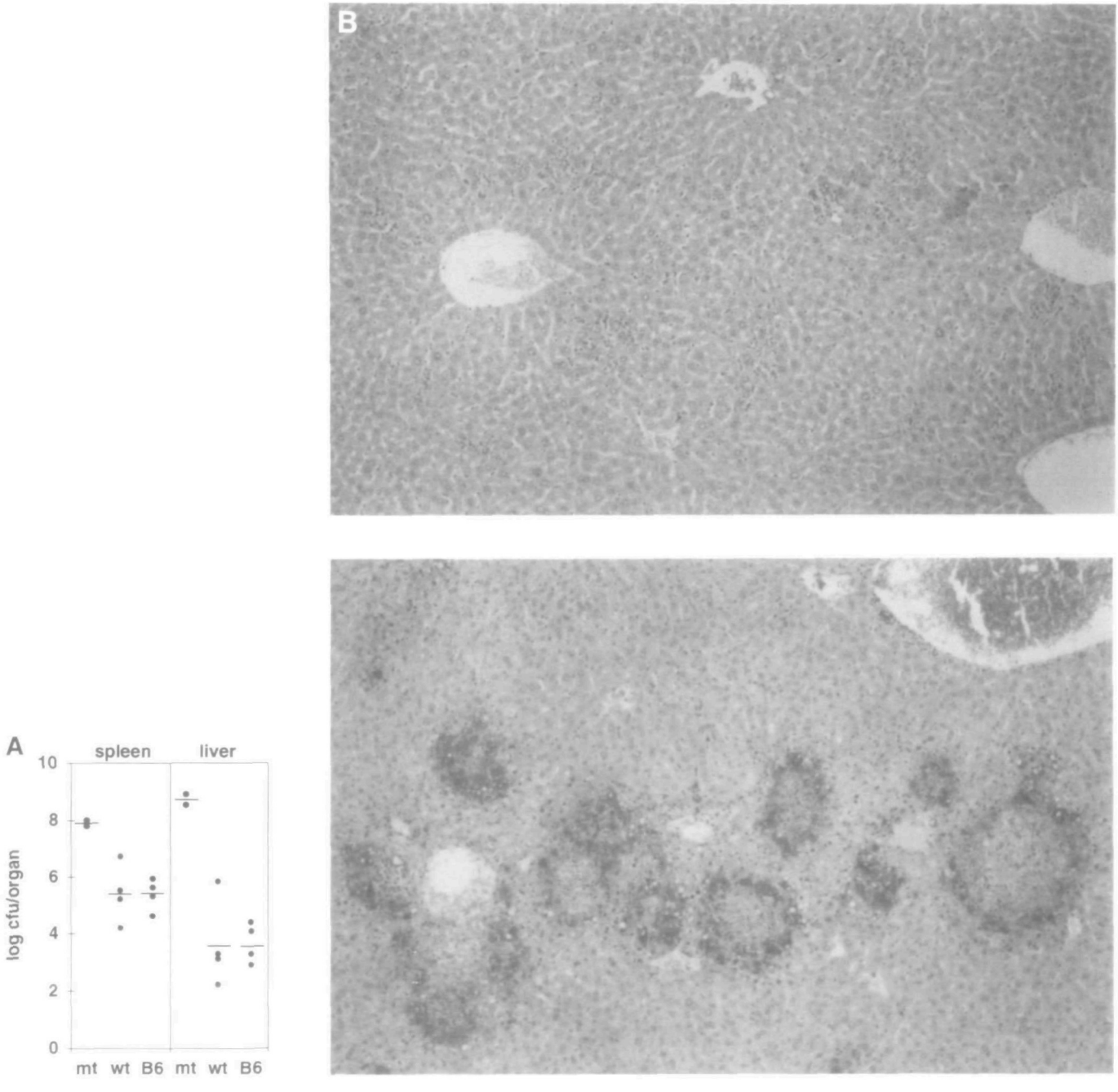

Fig. 8. (A) Liver and spleen listerial titers of mutant (mt), wild-type (wt) and C57BL/6 (B6) mice 3 days after i.v. infection with 300 c.f.u. of *L. monocytogenes* and (B) liver sections of wild-type (top) and mutant mice (bottom) showing extensive necrosis in mutant livers at day 6 after infection (×90).

any kind of pathology in a non-specific pathogen-free environment is currently under investigation Since not only TNF, but also other cytokines have been shown to be able to induce MHC class I expression, serum cytokine levels for IFN-γ, IL-1, IL-12 and transforming growth factor-β were determined, but no difference in cytokine levels could be demonstrated in the serum of mutant mice (not shown), which, however, does not exclude changes in the local production of these cytokines.

The general reduction of basal Ig levels is likely due to the absence of LN and PP The reduction in the basal Ig levels and the defective IgG isotype class switch is reminiscent of that seen in *aly*, CD40 and CD40L-deficient mice (41,53,54), and suggests a common defect in all three mutant mice provoked by different mutations in the same or convergent signaling pathways of isotype switching. The fact that CD40 ligation has been shown to induce surface LT-α on human B cells (55) and that TNF/LT-α-deficient mice show an isotype switch deficiency with the T-cell-dependent antigen SRBC, strongly suggests a co-stimulatory function of LT-α in T–B cell interaction necessary for efficient T-cell-dependent isotype switching

TNF and LT-α can protect against infections with RNA and DNA viruses (26–29). The anti-viral responses are either humoral and/or cytotoxic In our experiments we used VSV to investigate the humoral and VV and LCMV viruses to test the virus-specific cytotoxic T cell responses. While the antibody response to SRBC revealed a specific IgG class switch defect, the neutralizing IgM and IgG response against VSV was normal in mutant mice The humoral response against a broad range of viral infection in mice is IgG2a restricted (56), an isotype response which might be conserved in the mutant mouse

In contrast to the present neutralizing immune response, the primary MHC class I restricted *ex vivo* CTL responses are strongly reduced for LCMV-ARM and LCMV-WE In comparision, the CTL responses in TNFR1-deficient mice were normal (17) and no abnormal CTL response was reported for TNFR2-deficient mice (36). This suggests that signaling via the LT-β-receptor might be important for the primary *ex vivo* CTL responses. It has been shown that TNF is not involved in the acute (short-term) target cell damage induced by CTL and a reason for this seems to be the down-regulation of TNFR1 expression preceding TNF and LT-α secretion in the effector phase (57,58). The reduction observed in our mutant mice might stem from the inefficient activation of T_h cells or precursor CTL due to the absence of a LT-α/LT-β-receptor mediated co-stimulatory signal or due to low IL-2R expression of activated precursor CTL. Observations supporting the latter assumption are the IL-2R α chain induction by TNF and the suppression of the *in vivo* priming for TNP-specific CTL by anti-TNF antibodies (59,60). The interpretation of these *in vitro* data is, however, related to the finding that IL-2-deficient mice exhibit only a marginal reduction of the primary LCMV-specific CTL response, but clear the virus similar to the TNF/LT-α-deficient mice (61). The fact that secondary CTL responses are present, but still reduced compared with control mice, for both LCMV strains suggests the presence of virus-specific precursor CTL, which, however, require prolonged activation to become competent effector cells. Analysis of secondary CTL 13 and 20 days after i.v. priming resulted in loss of LCMV-WE-specific CTL from mutant mice, which is probably the result of virus-specific CTL exhaustion due to fast replication within the lymphatic and non-lymphatic environment (62). In any case, mutant mice were still able to clear the viruses from spleen and liver. Infection of LCMV-ARM as well as LCMV-WE into the footpad did not provoke footpad swelling, which reflects the absence of CTL-mediated immunopathology. Nevertheless, footpad injection of LCMV-ARM lead to a potent secondary CTL response in contrast to LCMV-WE. The lack of the secondary CTL response towards LCMV-WE after footpad infection is likely due to CTL exhaustion. The fact that footpad infection with LCMV-ARM lead to an efficient secondary CTL response demonstrates that a central response after a peripheral challenge is possible also in the absence of LN. We suggest that the lack of footpad swelling, which is a delayed type hypersensitivity reaction, must be due to impaired recirculation of memory CTL. This impairment is likely due to the aberrant splenic microarchitecture and might therefore be the reason for the absence of peripheral immunopathology

Infection with the Gram-positive, facultative intracellular bacterium *L monocytogenes* has been shown to induce cell-mediated immunity and efficient clearance of this pathogen critically depends on proper T cell and macrophage function (63,64) Genetic inactivation of TCRα, TCRβ, MHC class I and II, IFN-γR, TNFR1 and IL-6 (reviewed in 65) has defined a critical network of cytokines which play a crucial role in macrophage activation, extravasation and granuloma formation. The sensitivity towards low titer infection with *L. monocytogenes* seen in the TNF/LT-α-deficient mice is comparable with that of TNFR1-deficient mice and confirms a crucial role for TNF in the defence against *L monocytogenes* infections The fact that perforin (66) and MHC class I deficiency (67) does not lead to a drastic increase in sensitivity to primary *L monocytogenes* infection shows that other cell populations than T cells, like neutrophils and macrophages (64,68), are critical for an efficient primary host defence against *L. monocytogenes*. The anti-listerial activity of mutant macrophages might be impaired at least at two levels. First, the lack of TNF, which is an important co-stimulator of IL-12 for IFN-γ production by NK cells, might lead to a decreased activation of macrophages and a reduced development of an efficient T_h1 response (69,70). Second, phagocytosed *L. monocytogenes* might not be killed efficiently. The second assumption is supported by the fact that IFN-γ stimulated BMDM from mutant mice showed reduced *in vitro* killing of phagocytosed *L. monocytogenes* compared with control macrophages (not shown).

In conclusion, the combined genetic inactivation of TNF and LT-α leads to distinct morphological and functional defects of the peripheral immune system whereby absence of TNF and LT-α markedly affects innate and acquired immunity, respectively. The described phenotypic features render these mice interesting tools to investigate in more detail the roles of both ligands for the development and function of the immune system. The interchangability of TNF and LT-α still leaves open questions, mainly about the functions of the

soluble form of LT-α, which might be answered if a TNF knockout mouse would become available.

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Abbreviations

CTL	cytotoxic T lymphocytes
D-GalN	D-galactosamine
ES	embryonic stem cells
LPS	lipopolysaccharide
LCMV	lymphocytic choriomeningitis virus
LT	lymphotoxin
LN	lymph nodes
PP	Peyer's patches
SRBC	sheep red blood cells
TNF	tumor necrosis factor
VV	Vaccinia virus

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